Induction and Patterning of the Cement Gland
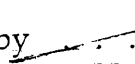during *Xenopus laevis* Embryogenesis

by

Brenda Schafer Kennedy

Submitted to the Department of Biology
in Partial Fulfillment of the Requirements for the
Degree of

Master of Science

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Signature of Author . . . . . . . . . . . . . . . . . . . . . . . . . . .
Department of Biology
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Certified by . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hazel L. Sive, Associate Member of the Whitehead Institute
for Biomedical Research and Assistant Professor of Biology
Thesis Advisor

Accepted by . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Frank Solomon, Graduate Committee Chairman
Department of Biology

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## ABSTRACT

The cement gland is a secretory organ that is the most anterior structure in
the developing *Xenopus laevis* embryo. Despite its apparent simplicity, the
induction and patterning of the cement gland is a complex process involving
multiple tissue interactions. The initial step in cement gland induction is
mediated by signal(s) from the dorsal mesoderm which can induce the
expression of cement gland specific genes in the dorsal ectoderm. This
induction takes place during gastrulation and can occur in vitro when the
mesoderm is either underlying (appositional induction) or adjacent (planar
induction) to the ectoderm. Planar signals alone can specify cement gland
tissue in the appropriate anteroposterior position with respect to neural
tissue. Anteroposterior patterning within the cement gland itself can be seen
by the localized expression of the gene XA-1. Microdissection experiments
provide evidence that this patterning occurs later in development during
neurula stages and may be influenced by both suppressive and inductive
signals from other tissues.

Thesis Supervisor: Dr. Hazel L. Sive
  Title: Associate Member of the Whitehead Institute
    for Biomedical Research
    Assistant Professor of Biology

## ACKNOWLEDGEMENTS

**INTRODUCTION**

The developing amphibian embryo displays a complex pattern of dorsal structures which can be visualized both morphologically and by the regionalized expression of different genes. It is known that inductive interactions and morphogenetic movements (such as gastrulation) play key roles in the establishment of the anteroposterior axis (Slack and Tannahill, 1992). By the end of gastrulation, before tissues have differentiated, several regions of the anterior dorsal ectoderm have been specified (Sive et. al., 1989). This supports the idea that the anteroposterior axis is organized primarily during gastrulation, and an extensive amount of patterning may be present prior to neurula stages. There are several theories as to which types of tissue interactions may be involved in induction and patterning of the dorsal ectoderm. Two of these theories, first advanced by Spemann (1938), propose ways in which inductive signals may pass from the mesoderm to the ectoderm. One way is by appositional induction, whereby signals emanating from the involuted mesoderm instruct the overlying ectoderm to adopt a variety of fates (reviewed in Slack, 1991; Sharpe and Gurdon, 1990). Secondly, it has also been shown that inductive signals can pass from the dorsal mesoderm in a region where it is continuous with the ectoderm, a process known as planar induction (Dixon and Kintner, 1989; Doniach et. al., 1992). There is also evidence that cells induced to enter the neural pathway in turn induce neighboring cells to do the same, a process known as homeogenetic induction (Grunz, 1990; Servetnick and Grainger, 1991; Itoh and Kubota, 1991). In all of these studies the presence of induced neural tissue has been assayed either morphologically or by the expression of

neural-specific genes. Since most of these markers are not expressed until neurula stages and later, they do not allow direct analysis of the inductive events that occur earlier during gastrulation. Therefore, the ideal tissue to be used in these studies is one which, like neural tissue, is induced by dorsal mesoderm and is characterized by specific genes which are expressed at the time of this induction. The cement gland satisfies both of these criteria.

The cement gland is an organ derived from the ectoderm and has the distinction of being the most anterior structure in the developing *Xenopus laevis* embryo. It is one of the first identifiable structures to arise from the dorsal ectoderm, initially recognizable as a band of darkly pigmented cells in neurula stage embryos which compact during development to form a cone-shaped structure of elongate cells in hatching stage embryos (Nieuwkoop and Faber, 1967). It is functional during these stages as a mucus secreting organ which allows the embryo to attach to a solid support before it can feed and swim, after which it undergoes programmed cell death.

The cement gland is a an excellent marker of anterior, head specific differentiation. It is a very simple organ which arises primarily from the outer layer of the ectoderm (Drysdale and Elinson, 1992) and is believed to consist of a single cell type (Perry and Waddington, 1966; Lyerla and Pelizzari, 1974). Like neural tissue, the cement gland can be induced by dorsal mesoderm, and endogenously this induction occurs early in gastrulation (Sive et. al., 1989). The simplicity of the cement gland as compared to neural tissue means that the cement gland offers an ideal system to study the inductive interactions which contribute to the anteroposterior patterning of the ectoderm. It has been shown that both mesoderm produced by in vitro induction with activin (Cooke et. al., 1987) and dorsal mesoderm (Sive et. al.,

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1989) can induce cement gland tissue in naive ectoderm (i.e., by appositional induction). Cement glands are also observed in exogastrulae along with the expression of some neural-specific genes (Kintner and Melton, 1987; Dixon and Kintner, 1989; Ruiz i Altaba, 1990, 1992), suggesting that it can be induced in a planar arrangement as well. These studies support the notion that the cell interactions and signalling pathways that mediate induction of neural tissue may also play a role in cement gland induction.

Molecular markers expressed in the cement gland have been previously isolated (Sive et. al., 1989; Jamrich and Sato, 1989). Two of these markers are XAG-1, which has previously been shown to be primarily cement gland specific and XA-1, which is expressed in the cement gland as well as the more posterior hatching gland (Sive et. al., 1989; Hemmati-Brivanlou et. al., 1990). XAG-1 and XA-1 are first expressed during gastrulation, when induction of the dorsal ectoderm is thought the occur, and continue to be expressed later in development in differentiated organs. Therefore, these markers provide a molecular means of analyzing both the early induction of the ectoderm as well as some of the later cell-cell interactions that may play a role in defining the anteroposterior characteristics of the dorsal ectoderm.

This current study includes a detailed analysis of the expression patterns of XAG-1 and XA-1 by *in situ* hybridization, which allowed visualization of cement gland formation during normal development. The use of these markers has also facilitated analysis of the steps involved in cement gland induction. This induction occurs during gastrulation and proceeds vertically through the ectoderm, with first the inner and later the outer layer of the ectoderm specified to express XAG-1 and XA-1.

Differentiation of cement gland tissue can also occur in explants where appositional mesoderm/ectoderm contacts are abolished. Moreover, this planar signalling allows development of the cement and hatching glands in the appropriate anteroposterior position in these explants with respect to neural tissue. Finally, the expression pattern of XA-1 has shown the presence of anteroposterior polarity within the cement gland itself. This polarity is not present during gastrulation when the cement gland is first induced, but is established later in development and may require both positive signalling from more posterior ectoderm as well as inhibition which may be mediated by more ventral tissues. Taken together, these data suggest a multi-step process in cement gland induction and patterning.

## MATERIALS AND METHODS

### Growth and culture of embryos

Embryos were collected from adult *Xenopus laevis* females after injection with human chorionic gonadotropin (700 - 1000 USP Units) and in vitro fertilized with dissected testis. Embryonic staging was according to Nieuwkoop and Faber (1967). Embryos were grown in 0.1X modified Barth's saline (1XMBS: 88mM NaCl, 1mM KCl, 1mM CaCl$_2$, 1m MgCl$_2$, 5mM Hepes, 50 mg/mL gentamycin; pH 7.8) at room temperature or 15$^{\circ}$C. Embryos were dejellied in 2% cysteine (pH 7.6) followed by extensive washing in 0.1X MBS.

### Plasmids

cDNA clones used as probes for XA-1, XAG-1 and XCG-1 were EcoRI fragments subcloned into pBStSK+ (Stratagene) (Sive et. al., 1989). A 930bp

EcoRI insert subcloned into pBStSK- (Stratagene) was used as a probe for UVS.2 (Sato and Sargent, 1990). For *en-2* analysis, a 3kb insert subcloned into pSP65 was used (Hemmati-Brivanlou et. al., 1991). Detection of EF-1a was performed using a 375bp insert in pGEM1 (Krieg et. al., 1989).

**Dissections**

After dejellying, the vitelline membranes were removed from embryos with watchmaker's forceps. The embryos were dissected using tungsten needles in 60mm petri dishes coated with 1% agarose. Dissections and inductions of embryos and explants were performed in 1X Holtfreter's solution (60mM NaCl, 0.7mM KCl, 0.9mM $CaCl_2$, 25mM $NaHCO_3$, 10 mg/mL penicillin/streptomycin; pH 7.5) which included 10mM $NH_4Cl$ for inductions. Following induction for 6 hours, embryos and explants were washed and allowed to develop in 1X Holtfreter's solution.

Dissection and incubation of St. 11.5 dorsal ectoderm and dorsal ectoderm plus underlying mesendoderm was done in 1X MBS, whereas layer separation experiments were done in 0.5X MBS.

Keller sandwiches were prepared as previously described (Keller and Danilchik, 1988; Doniach, 1992) and embryos were dissected in Sater's modified blastocoel buffer (53mM NaCl, 32mM NaGluconate, 5mM $Na_2CO_3$, 4.5mM KGluconate, 1mM $CaCl_2$, 1mM $MgSO_4$, 50 mg/mL gentamycin; pH 8.2). Rectangular pieces of tissue from the dorsal side of St. 10+ embryos were dissected. These pieces of tissue included the dorsal involuting marginal zone (DIMZ), the non-involuting marginal zone (NIMZ) and a portion of the animal cap extending approximately to the animal pole. The dorsal lip and any involuted head mesoderm were removed. Two explants were

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assembled with inner surfaces facing each other and were gently flattened under a glass coverslip to prevent the explants from curling up. Explants were incubated in Sater's modified blastocoel buffer.


## Northern Analysis

RNAs were isolated from whole embryos/explants by homogenizing dissected tissue, followed by proteinase K digestion (500ug/mL), phenol extraction, and EtOH precipitation (Condie and Harland, 1987). Samples were loaded onto 1.5% agarose/MOPS/formaldehyde gels which were blotted to Hybond-N nylon membrane (Amersham) and UV crosslinked. Northern blots were hybridized with antisense probes made by asymmetric amplification by PCR from either inserts or linearized templates, as described in Sive and Cheng, 1991.


## In Situ Hybridization

Embryos were fixed and processed for in situ hybridization as previously described (Harland, 1991). Explants received a reduced exposure time to proteinase K digestion as compared to whole embryos. RNA probes were prepared by in vitro transcription from linearized plasmid templates using DNA-dependent RNA polymerase (SP6, T3, or T7) in the presence of digoxygenin-11-UTP (Boehringer Mannheim). Probes were hydrolyzed to approximately 300-400nt fragments. Embryos/explants were hybridized with 0.5 to 1ug of probe at 60°C for at least 16 hours. Hybridization of probe was assayed by using an alkaline-phosphatase coupled antibody to digoxygenin (Boehringer Mannheim; diluted 1:2000), followed by colorimetric detection of alkaline phosphatase in the presence of NBT (p-nitro blue tetrazolium

chloride) and BCIP (5-brom-4-chlor-3 indolyl phosphate).

**RESULTS**

**Patterns of XAG-1 and XA-1 expression during development**

XAG-1 and XA-1 cDNAs were isolated in a subractive hybridization scheme
(Sive and St. John, 1988) designed to identify head-specific transcripts. The
temporal and spatial expression of each transcript was determined by
dissection and Northern analysis (Sive et. al., 1989). XAG-1 is a 2kb transcript
that is first detectable in early gastrula stages and persists until after the
cement gland has degenerated. XA-1 is first expressed near the end of
gastrulation and continues to be expressed until the hatching and cement
glands degenerate. Specific localization of these transcripts in developing
embyos was determined by *in situ* hybridization (Hemmati-Brivanlou et. al.,
1990). These studies confirmed that XAG-1 transcripts are primarily localized
to the cement gland whereas XA-1 is expressed in both the hatching gland
and cement gland in tailbud and hatching stage embryos.

XAG-1 and XA-1 expression patterns in whole embryos were
visualized in more detail by *in situ* hybridization (Harland, 1991) using
digoxygenin-labeled anti-sense probes. Control experiments using sense
probes in parallel hybridizations were performed to confirm that expression
patterns seen for each gene were specific (data not shown).

XAG-1 expression in whole embryos is first detectable at the end of
gastrulation (st. 12.5, Fig. 1A). At this stage XAG-1 RNA is localized to a
group of ectodermal cells in the vicinity of the anterior neural plate. This
region of the ectoderm corresponds to presumptive cement gland tissue.

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Figure 1. Developmental expression of XAG-1, XCG-1, XA-1 and UVS.2 by whole mount *in situ* hybridization.
In all panels except (H), frontal views are shown with dorsal on top. Panel H is a dorsal view with posterior on top. (A, B, C, D) show expression of XAG-1; (E, F, G): XCG-1; (H, I, J, K): XA-1; and (L, M, N): UVS.2. All magnifications are 25X.

(A) XAG-1 expression at late gastrula stage (st. 12.5). Staining is seen in a region anterior to the neural plate. (B) By mid-neurula stage (st. 17), XAG-1 is expressed in the developing cement gland, whose boundaries have sharpened. (C, D) XAG-1 continues to be expressed in the cement gland at tailbud (C: st. 24) and hatching (D: st. 33) stages and is also expressed at a low level in the more posterior hatching gland (arrow in C, D). (E,F,G) XCG-1 expression is confined to the cement gland throughout development. XCG-1 is first expressed at mid-neurula stage (E: st. 17) and is localized to a similar subset of cement gland cells as XAG-1 at this and later stages (F: st. 24; G: st. 33). (H) Dorsal view of XA-1 expression at early neural stage (st. 14). Arrows indicate expression in two regions at the lateral edge of the anterior neural plate. (I) Following neural tube closure (st. 17) XA-1 is expressed in the presumptive hatching gland and at a low level at the posterior edges of the cement gland (arrow). (J, K) XA-1 continues to be expressed in the hatching gland during tailbud (J: st. 24) and hatching (K: st. 33) stages. Expression in the cement gland has extended but is still confined to the posterior region. (L, M, N) UVS.2 expression is hatching gland specific. Expression is first detected during neurula stages (L: st. 17). UVS.2 is expressed in the same subset of hatching gland cells as XA-1 at later stages (M: st. 24; N: st. 33).
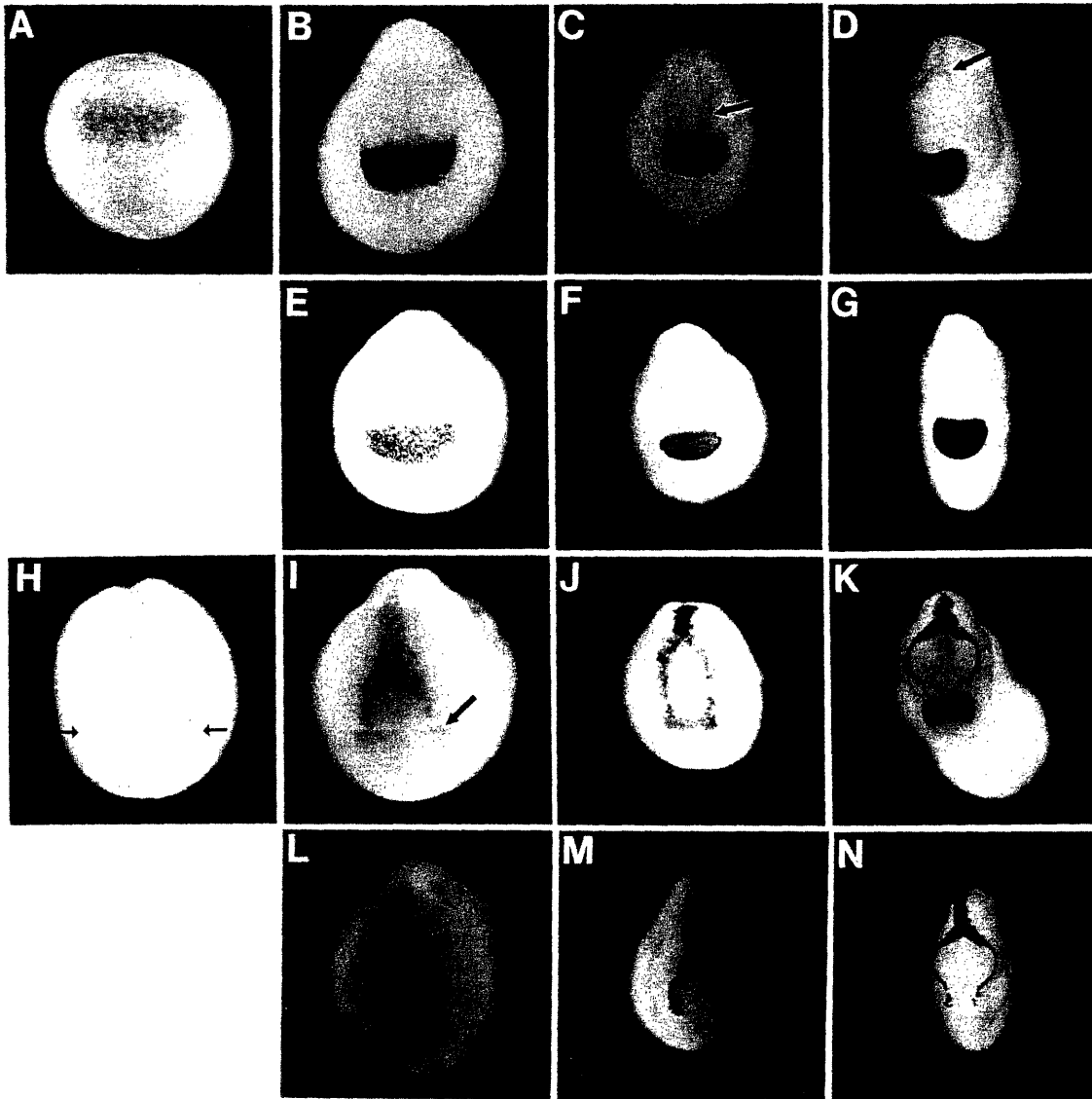
XAG-1 is clearly expressed in the outer layer of cells in the ectoderm at this stage; sectioning of the embryos would be necessary to determine if it is also expressed in the inner layer as well. The border of expression of XAG-1 becomes more distinct in early neurula embryos (st. 17, Fig. 1B), possibly due to the convergence of the cells that contribute to the developing cement gland (Lyerla and Pellizzari, 1974). During neurula stages the developing cement gland (and XAG-1 expression) is confined to a region anterior to the neural plate and dorsal to the anterior border of the involuted ventral mesoderm. As development proceeds to tailbud stages (st. 24, Fig. 1C) XAG-1 continues to be expressed in the cement gland, but is also detected more posteriorly, in a subset of cells which make up the developing hatching gland. XAG-1 expression in the cement gland persists through hatching stages (st. 33, Fig 1D) when the gland cells are organized into a columnar epithelium and secrete mucus (Picard, 1975a; Perry and Waddington, 1966).

To confirm that XAG-1 is expressed in presumptive cement gland cells during development, expression of a cement-gland specific transcipt was assayed in a parallel set of embryos. XCG-1, which was isolated in the same subtractive scheme as XAG-1 and XA-1, is expressed only in cement gland cells throughout development, based on Northern analysis of dissected embryos (Sive et. al., 1989). Unlike XAG-1, which is first expressed during gastrulation, XCG-1 is not detectable until mid-neurula stages (st. 17, Fig. 1E). It is around this time that cement gland cells are first identifiable morphologically as a group of cells darkened by pigment granules (Nieuwkoop and Faber, 1967). Comparison of XAG-1 and XCG-1 localization in embryos at these and later stages (compare Figs. 1B, C, D to Figs. 1E, F, G) suggests that XAG-1 is expressed in the same subset of cells in the developing

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cement gland as XCG-1. However, the subcellular localization of these two transcripts differ: in the differentiated cement gland XCG-1 is expressed throughout the elongate cells whereas XAG-1 is primarily expressed in the basal portion of the cells (data not shown). Later in development before the cement gland has degenerated, the pattern of XAG-1 expression differs from XCG-1, which is still detectable throughout the cement gland (st. 41, Figs. 2A, 2B ). At this and later stages XAG-1 is expressed in other tissues of the developing embryo including the gut (Fig. 2D) and in a region of the head between the brain and the branchial arches (Fig. 2E).

XA-1 expression is first detected in whole embryos as two narrow strips near the lateral and anterior boundary of the neural plate (st. 14, Fig. 1H). Posteriorly, these two regions of XA-1-positive cells are fused along the dorsal midline following neural tube closure (st. 17, Fig. 1I). Anteriorly the expression diverges from the midline to two lines of XA-1 expressing cells, terminating in the region of the presumptive cement gland. This pattern of expression matches the description of the developing hatching gland (Sato and Sargent, 1990; Drysdale and Elinson, 1991). To confirm that XA-1 is expressed in presumptive hatching gland cells, parallel embryos were hybridized with a hatching gland-specific clone, UVS.2 (Sato and Sargent, 1990). It can be seen that throughout development both UVS.2 and XA-1 are expressed in a similar subset of hatching gland cells (compare Figs. 1I, J, K to Figs. 1L, M, N). However, unlike UVS.2, as early as mid-neurula stage, XA-1 is not confined to the hatching gland but is also detected at a low level in the posterior region of the cement gland (Fig. 1I). The initial staining in the cement gland is in the cells that are adjacent to the hatching gland. This early expression of XA-1 in the cement gland appears to be in the deep inner

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Figure 2. Expression of XAG-1, XCG-1 and XA-1 in swimming tadpoles by whole mount *in situ* hybridization. All magnifications are 25X.

(A) Frontal view (dorsal on top) showing decreased expression of XAG-1 in the cement gland at swimming tadpole stage (st. 41) as compared to XCG-1 (B) which is still expressed throughout the cement gland. (C) After degeneration of the hatching gland XA-1 is still expressed in the cement gland and remains localized to the posterior region (st. 41). (D) Side view of a tadpole (anterior to the right, dorsal on top) showing that by st. 43 XAG-1 is expressed in a restricted region of the developing gut (open arrow) and in the head (closed arrow). (E) Ventral view of dissected tadpole at the same stage as (A) where tail and gut have been removed to visualize staining in the head (open arrow). A low level of XAG-1 is seen in the basal region of the cement gland (closed arrow). Anterior is to the right.
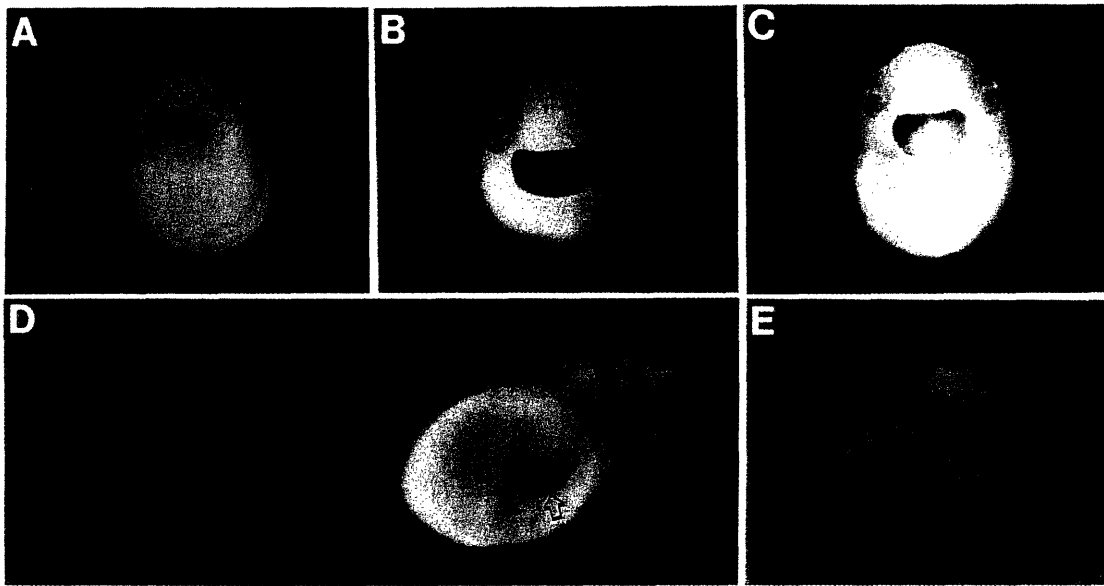
layer of the ectoderm.  By tailbud stage (st. 24, Fig. 1J) XA-1 RNA is present in

both the hatching gland and the cement gland, although at this stage it's

expression in the latter is detected in the outer, surface layer of cells and is

confined to the posterior region of the cement gland.  This asymmetry of XA-

1 transcripts in the cement gland persists until hatching stages (st. 33, Fig. 1K).

In later stages of development after the hatching gland has degenerated, XA-1

is still expressed at a low level in the posterior portion of the cement gland

(st. 41, Fig. 2C).  Unlike XAG-1, it is no longer expressed in the embryo

following degeneration of the cement gland (data not shown).


**Cement gland organization in NH4Cl induced ectoderm**

It has been shown that cement gland tissue can be induced in explants of

isolated competent ectoderm (blastula/early gastrula stage) which are treated

with 10mM NH4Cl and cultured in neutral saline to st. 35 equivalent (Picard,

1975b).  These explants go on to form cement gland tissue which is

recognizable both morphologically (Picard, 1975c) and by the expression of

cement gland specific genes (Sive et. al., 1989; Jamrich and Sato, 1989).

Additionally, NH4Cl treated ectoderm can be "reprogrammed" to express

neural markers after conjugation with dorsal mesoderm.  NH4Cl treatment

prolongs the competence of the ectoderm to respond to mesodermal signals,

since isolated ectoderm that is not treated but aged to an equivalent stage as

NH4Cl induced ectoderm is no longer responsive to induction by mesoderm

(Sive et. al., 1989).

To analyze the organization of in vitro induced cement glands in

more detail, animal caps from late blastula (st. 10) embryos were dissected

and placed in 1X Holtfreter's solution, with or without 10mM NH4Cl, for six

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hours. Following NH4Cl treatment, the explants were washed extensively and allowed to develop in neutral saline to st. 30 equivalent, when control embryos had developed functional cement glands (Fig. 3A). The expression of cement gland markers in these explants was analyzed both by Northern blot and *in situ* hybridization. Northern analysis shows that neither XAG-1 nor XA-1 are expressed in explants incubated in 1X Holtfreter's alone (Fig. 3B, lane 2), but both of these genes are induced in NH4Cl treated explants (Fig. 3B, lane 3). Levels of RNA loaded in each lane were determined by monitoring the expression of EF-1a, a ubiquitous transcript (Krieg et. al., 1989). Whole embryos treated with NH4Cl are indistinguishable morphologically from control untreated embryos. The relative abundance of XAG-1 and XA-1 RNAs in these embryos is also similar (Fig. 3B, compare lanes 1 and 4).

The expression of XAG-1, XA-1 and XCG-1 in these explants was assayed by *in situ* hybridization, to determine the extent of differentiation of cement gland tissue in NH4Cl induced animal caps. Untreated animal caps go on to form disorganized masses of atypical ciliated epidermis characterized by rough, textured surfaces (Holtfreter, 1945). None of the explants analyzed express XAG-1 (Fig. 3C, panel A), XA-1 or XCG-1 (data not shown). NH4Cl treated animal caps are smoother than untreated caps, and sometimes appear more elongate. Picard (1975c) has shown that in vitro induced cement glands are structurally and histochemically similar to natural cement glands. XAG-1 and XCG-1 are expressed in the majority of induced caps analyzed (15/16 for XAG-1; 10/11 for XCG-1). However, induction of cement gland cells in NH4Cl treated animal caps is somewhat variable (Picard, 1975b,c). This can be seen in induced caps hybridized with

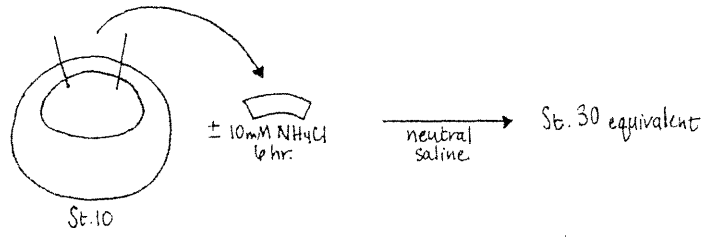Figure 3. NH$_4$Cl induction of cement gland

(A) Induction scheme. Sagittal section of a late blastula is shown. Stage 10 animal caps were cultured in 1X Holtfreter's solution with or without 10mM NH$_4$Cl for six hours at room temperature. Explants were washed and allowed to develop in 1X Holtfreter's until control embryos reached stage 30 equivalent. Control embryos were treated in a similar fashion. Explants were either harvested for Northern analysis or fixed for *in situ* hybridization.
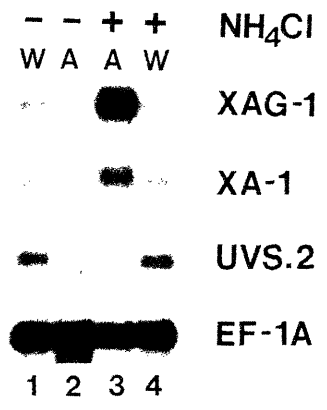
(B) Northern analysis of embryos and explants harvested at stage 30 equivalent. W = whole embryo, A = animal cap. Pluses and minuses above the lane indicate which embryos and explants received the NH$_4$Cl treatment. Lanes 1 and 4 contain one embryo equivalent per lane. Lanes 2 and 3 contain 10 explants per lane.

(C) In situ hybridization of explants. (A) Animal caps treated continuously with 1X Holtfreters and probed with XAG-1. (B) XAG-1 expression in caps treated with NH$_4$Cl. XA-1 expression (C) and XCG-1 expression (D) in NH$_4$Cl treated animal caps. Magnification is 40X.

**A**



St. 10    ± 10mM NH₄Cl  6 hr.    neutral saline    St. 30 equivalent

**B**



| − | − | + | + | NH₄Cl |
| W | A | A | W | |

XAG-1

XA-1

UVS.2

EF-1A

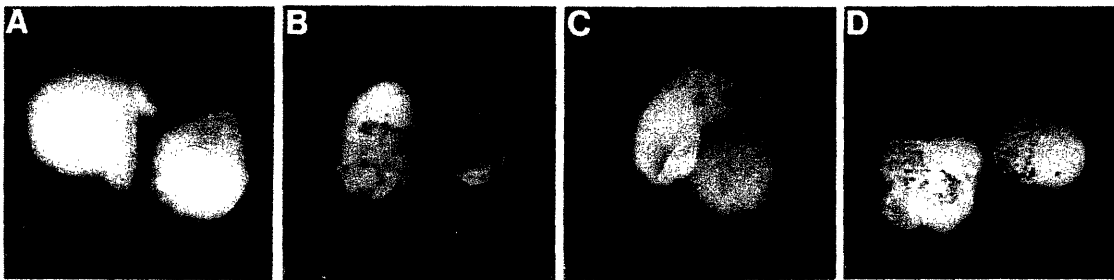1  2  3  4

**C**



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XAG-1 and XCG-1 probes (Fig. 3C, panels B, D). In some cases most of the cells express XAG-1 or XCG-1 whereas in other explants there are a small number of positive cells. Also, there are non-expressing cells mixed within a population of expressing cells. This is never seen in the natural cement gland. XA-1 expression in NH4Cl induced cement glands is very different from what is seen in the cement glands of whole embryos. Only a subset of the induced caps express XA-1 (9/13) and this expression is not spatially restricted as it is in the cement gland of the embryo (Fig. 3C, panel C). The level of XA-1 expression detected in the NH4Cl induced animal caps is less than expected based on Northern analysis. This may be due to expression in some cells that is too low to be detected by *in situ* hybridization.

Since XA-1 is expressed in both the cement gland and the hatching gland, it is a possibility that XA-1 expression in NH4Cl induced animal caps is not specific to cement gland tissue, but may be the result of hatching gland also being induced in these explants. Two lines of evidence suggest that this is not the case. First, there is no expression of the hatching gland specific clone UVS.2 in NH4Cl induced animal caps, as seen by Northern analysis (Fig. 3B, lane 3). Also, the levels of XAG-1 and XA-1 in NH4Cl induced animals caps were compared to expression in the whole embryo or in isolated cement glands dissected from embryos of an equivalent stage (st. 22) as the induced caps (Fig. 4A). The level of XA-1 is higher in whole embryos (Fig. 4B, lane 1), since these include both cement gland and hatching gland tissue, whereas the relative concentrations of XAG-1 and XA-1 are similar in NH4Cl-induced tissue and in cement gland isolated from whole embryos (Fig 4B, lanes 2 and 3). There is no UVS.2 expression in the isolated cement glands, suggesting that the dissected tissue was not contaminated with

Figure 4.  XAG-1 and XA-1 expression in normal versus NH4Cl induced cement glands.

(A) Scheme of dissection and inductions.  St. 10 animal caps were cultured in 1X Holtfreter's solution with 10mM $NH_4Cl$ for six hours at room temperature.  Explants were washed and allowed to develop in 1X Holtfreters until control embryos reached early tailbud (st. 22) equivalent.  Cement glands were dissected from control embryos harvested at the same stage as the treated animal caps.

(B) Northern analysis of explants and embryos harvested at stage 22.  W = whole embryo, C = cement gland only, A = animal caps.  Lane 1:  RNA isolated from untreated control embryos, one embryo equivalent; lane 2: dissected cement glands from untreated embryos, one cement gland; lane 3: animal caps treated with $NH_4Cl$, 5 explants.

**A**
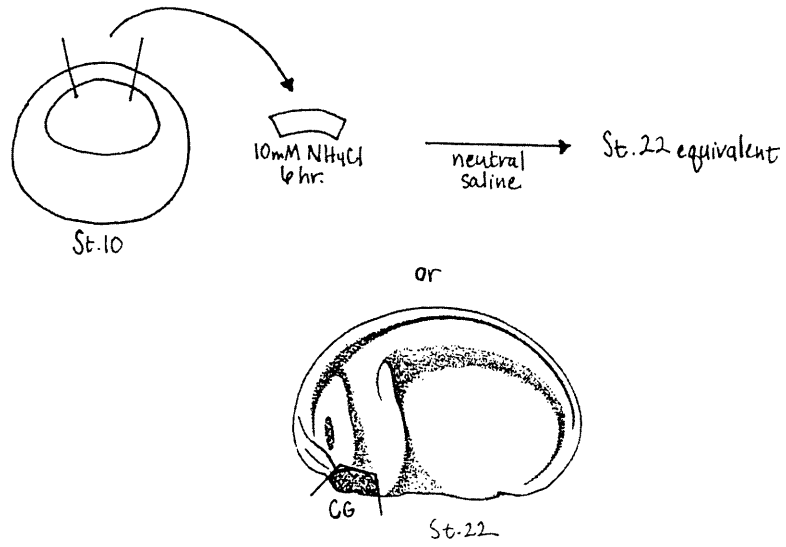


**B**
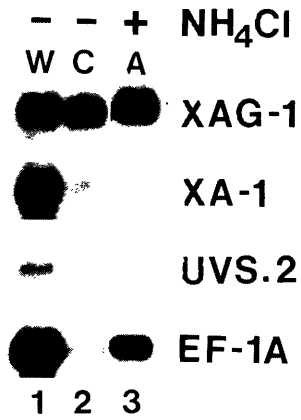
hatching gland tissue, which would increase the level of XA-1 RNA in the samples.

In summary, NH4Cl induced cement gland is similar to naturally induced tissue in terms of levels of gene expression. However, the organization of these artificially induced cement glands is qualitatively different, since the patterns of gene expression within these tissues are not the same as seen in cement glands of the whole embryo. This may reflect the fact that cell-cell interactions necessary for patterning the cement gland in the embryo are not present in NH4Cl induced cement glands.


**Early induction of cement gland is ectoderm layer specific**
XAG-1 and XA-1 are expressed during gastrulation when the initial anteroposterior patterning of the dorsal ectoderm is thought to occur (Sive et. al., 1989). In this regard, they provide useful markers for analyzing some of the early cell-cell interactions involved in induction of the ectoderm. It has been shown that dorsal ectoderm destined to become neural plate is induced to a "pre-cement gland" state early in gastrulation, and is subsequently respecified as neural tissue later in gastrulation (Sive et. al., 1989). This supports a model whereby the dorsal ectoderm is progressively patterned during gastrulation, with posterior tissue transiently induced as more anterior tissue.

By gastrulation the ectoderm is divided into two layers: a darkly pigmented outer, surface layer and a lightly pigmented inner, deep layer (Jones and Woodland, 1986). In an attempt to learn more about the early specification of the cement gland during gastrulation, the induction of XAG-1 and XA-1 in individual layers of the ectoderm was analyzed. Pieces of

ectodermal tissue underlain by involuted mesoderm were dissected from embryos at early gastrula (St. 10.5 to 11) or late gastrula (St. 11.5 to 12) stages (Fig. 5A). Any attached mesoderm was removed. The piece of ectoderm removed from the earlier stage embryos does not contribute to the mature cement gland, as shown by vital dye marking experiments (Sive et. al., 1989), whereas ectoderm excised at later stages includes presumptive cement gland cells. These "induced" pieces of ectoderm were either separated into inner and outer layers or left intact and allowed to develop in neutral saline until control embryos reached st. 28 equivalent. The state of induction of each tissue was determined by analyzing the levels of XAG-1 and XA-1 expression in pools of explants.
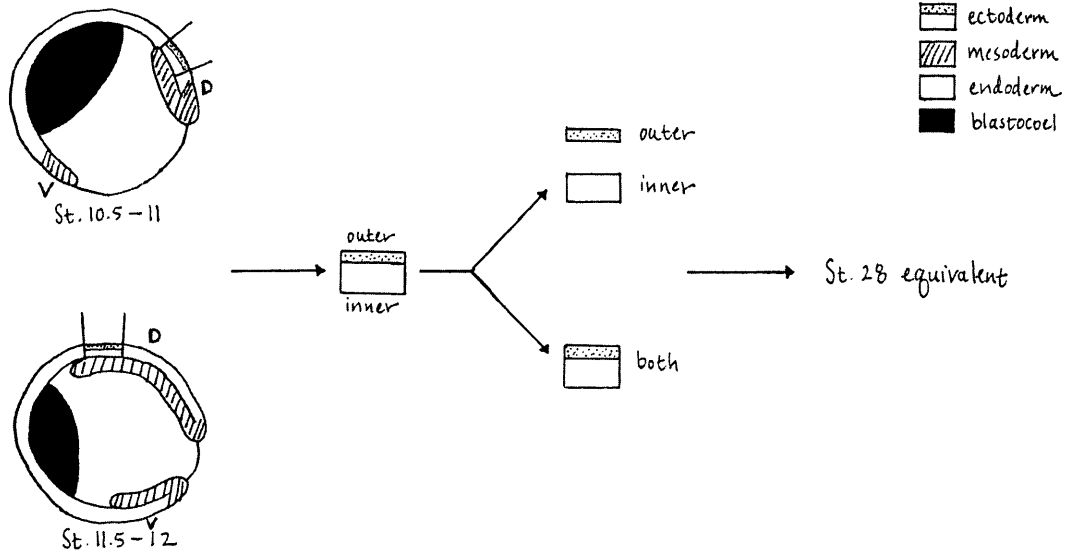
Early in gastrulation, XAG-1 is induced only in the inner layer of the ectoderm (Fig. 5B, lane 2). There is no detectable expression in the outer layer (Fig. 4B, lane 3) The level of XAG-1 RNA in inner layer explants is similar to that observed in explants of intact ectoderm (Fig. 5B, lanes 2 and 4), as there are twice as many inner layer explants loaded per lane as intact explants. However, it is not known which layer of the intact ectoderm is expressing XAG-1 in these explants. Explants dissected at late gastrula stages shows that XAG-1 expression has been induced in both the inner and outer layers (Fig. 5B, lanes 5 and 6). XA-1 is induced at a very low level in the inner layer only during early gastrula stages (Fig. 5B, lane 2), and is expressed at a higher level later in gastrulation, but is still confined to the inner layer (Fig. 4B, lane 5). Induction of XA-1 in the outer layer is detectable when the explants are isolated at very late gastrula stages (St. 12 to 12.5, data not shown). Previous studies have shown that the cells that contribute to both the differentiated cement and hatching glands are primarily derived from

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Figure 5.  Ectoderm layer-specific induction of cement gland

(A) Dissection scheme.  Sagittal sections of gastrula stage embryos, D = dorsal, V = ventral.  Ectoderm overlying involuted dorsal mesoderm was dissected at either early gastrula (stage 10.5 - 11) or late gastrula (stage 11.5 - 12) stages. Ectodermal pieces were either separated into individual inner and outer layers or left intact.  Dissection and incubation of explants was in 0.5X MBS. Explants were allowed to develop at room temperature until control embryos reached tailbud (st. 28) equivalent and then harvested for Northern analysis.

(B) Northern analysis of embryos and explants harvested at st. 28 equivalent. W = whole embryo, I = inner layer only, O = outer layer only, B = both layers left intact.  Stages when explants were dissected are indicated above the lanes. Lane 1 contains 0.5 embryo equivalent of control embryos.  Lanes 2, 3, 5 and 6 contain 5 explants per lane.  Lanes 4 and 7 contain 2.5 explants per lane.

## A



ectoderm
mesoderm
endoderm
blastocoel

St. 10.5 - 11

St. 11.5 - 12

outer

inner

outer

inner

both

St. 28 equivalent

## B



XAG-1

XA-1

EF-1A
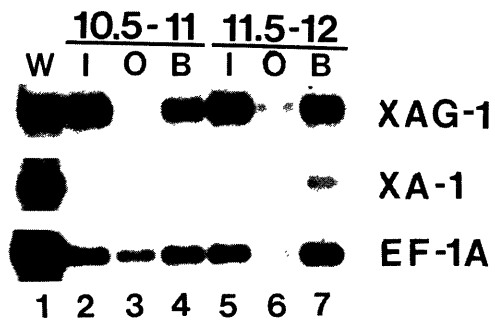
the outer layer of the ectoderm (Drysdale and Elinson, 1992). A possible criticism of the experiments presented here is that the outer layer of the ectoderm may be induced as well as the inner layer, but following dissection and separation from the inner layer it may not develop or differentiate in culture. This is not thought to be the case, since outer layer explants that have been isolated at late gastrula and allowed to develop to tailbud stages often display small, darkly pigmented cement glands (data not shown). This suggests that these explants do develop and differentiate in culture. Explants of intact ectoderm also routinely develop cement glands. Cement glands are not easily observed in inner layer explants, since the cells are lightly pigmented.

The results presented here suggest that there is a stepwise induction of the ectoderm - cells of the inner layer are initially specified and express cement gland markers, whereas cells in the outer layer are subsequently induced later in development. It is possible that more posterior ectoderm that is transiently induced to a "pre-cement gland state" only expresses XAG-1 in the inner layer prior to respecification. Perhaps only tissue destined to give rise to the natural cement gland secondarily induces the outer layer of the ectoderm, which may be an essential step in determination of the cement gland.


**Cement gland and hatching gland are induced by planar signals**
The previous results suggest that the involuted mesoderm may be vertically signalling the overlying ectoderm and specifying it as cement gland tissue. Inductive signals produced by the involuted dorsal mesoderm would come into contact with the inner layer of the ectoderm earlier than the outer layer,

and this could account for the stepwise induction that is observed. However, it is known that extensive morphogenesis and anteroposterior patterning of neural tissue can occur in Keller explants (Keller and Danilchik, 1988; Dixon and Kintner, 1989; Ruiz i Altaba, 1990; Doniach et. al., 1992). These explants allow signalling from the mesoderm to the ectoderm in a planar fashion but abolish vertical mesoderm/ectoderm contacts.

To determine whether planar signals alone can specify cement and hatching gland tissue, XAG-1 and XA-1 expression was observed in Keller sandwiches. Two equivalent pieces of tissue were dissected from the dorsal side of St. 10+ embryos and assembled with their inner surfaces apposed (see Materials and Methods; Fig. 6A). Signalling from the mesoderm to the ectoderm can occur only in a planar fashion at the posterior boundaries of these tissues, as the mesoderm is prevented from involuting under the ectoderm in these explants. Explants were cultured until control embryos reached st. 28, at which time they were assayed for the expression of specific genes by *in situ* hybridization.

In these explants, convergent extension of the mesoderm and ectoderm occurs as it does during gastrulation in whole embryos (Keller and Danilchik, 1988; Keller et. al., 1992). Elongation in the ectoderm is greatest in the posterior region, which gives rise to neural tissue (Keller et. al., 1992; Doniach et. al., 1992). In the Keller sandwich pictured in Figure 6B, panel C, XAG-1 is highly expressed in a band of cells around the circumference of the explant. These cells are believed to correspond to cement gland tissue, based on the observation that this general region secretes a mucus that causes the explant to stick to forceps. This pattern of staining in the cement gland has been observed previously for X-dll3, a distal-less homologue that is expressed
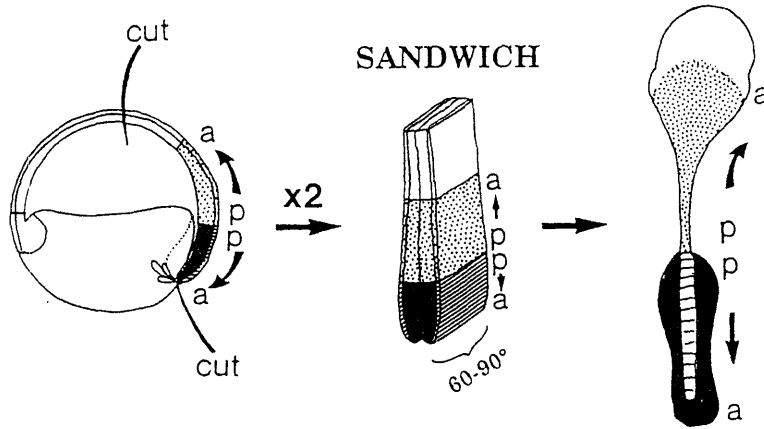
Figure 6. Induction of XAG-1 and XA-1 by planar signals.

(A) Diagram of a sagittal section of an early gastrula embryo (stage 10+) showing dissection of tissue for Keller sandwiches. Anteroposterior polarity of ectoderm and mesoderm is depicted. Normal fates in explanted region: white, epidermis; stippled, neurectoderm; black, chordamesoderm; striped, archenteron roof endoderm (Keller, 1975, 1976). Diagram adapted from Doniach, 1992.

(B) Expression of XAG-1, XA-1 and *en-2* in whole embryos and Keller sandwiches. In all panels anterior is to the left. Keller sandwiches were aged to an equivalent stage (st. 28) as control embryos. (A) and (C) show the expression of XAG-1 (closed arrow) and *en-2* (open arrow). *En-2* expression is posterior to XAG-1-expressing cells in both whole embryos and Keller sandwiches. (B) and (D) show the expression of XA-1 (closed arrow) and *en-2* (open arrow). XA-1 is expressed in the cement gland and hatching gland tissue in both embryos and Keller sandwiches. The relative anteroposterior positions of XA-1 and *en-2* are preserved in Keller sandwiches (D), and the posterior extent of XA-1 hatching gland expression extends posterior to *en-2* in both embryos and explants. Magnifications: 25X in (A) and (B); 40X in (C) and (D).

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# A



cut

SANDWICH

x2

60-90°

cut

a p p a

a p p a

a

p p

a

adapted from Doniach, 1992

# B



34
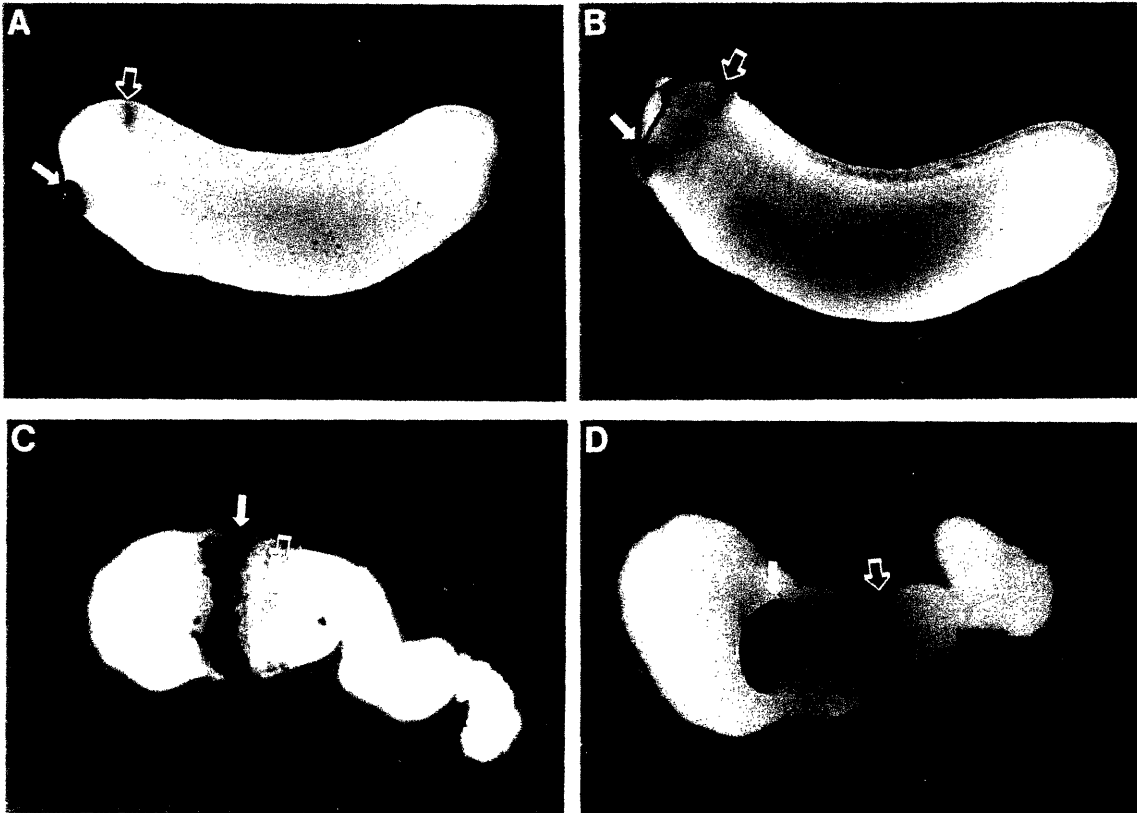
in anterior ectodermal derivatives of the developing embryo including the cement gland (Papalopulu and Kintner, 1993). Posterior to this heavy band of expression is a region which contains scattered XAG-1-positive cells. These cells are probably hatching gland cells, as XAG-1 is expressed at a low level in a subset of hatching gland cells at this stage in the whole embryo. Occasionally XAG-1 expression is seen in two distinct cement glands, presumably each derived from the individual dorsal ectoderm explants that make up the sandwich (data not shown).

To determine the relative anteroposterior position of the cement gland in these explants, XAG-1 expression was compared to the expression of a homeobox-containing gene, *engrailed-2* (*en-2*: Hemmati-Brivanlou et. al., 1991). *En-2* is localized in the developing central nervous system at the midbrain/hindbrain boundary of the brain, and it's expression pattern in Keller explants has been previously reported (Doniach et. al., 1992). The expression of *en-2* can be seen in st. 28 embryos (Fig. 6B, panel A, open arrow) in a narrow band of cells which are posterior to XAG-1 expression in the cement gland (Fig. 6B, panel A, closed arrow). The anteroposterior localization of these two genes is similar in Keller sandwiches, where *en-2* expression is confined to cells which are posterior to XAG-1-positive cement gland cells (Fig. 6B, panel C).

The differentiation of both cement and hatching gland tissues was further analyzed by observing XA-1 expression in Keller sandwiches. XA-1 expression is seen in both cement gland and hatching gland cells and, as shown in Fig. 6B, panel D, the organization of these tissues is very similar to that seen in the whole embryo (Fig. 6B, panel B). In the hatching gland, XA-1-positive cells form two lines that originate at the cement gland and extend

posteriorly beyond the limit of *en-2* expression. This can be seen in both explants and whole embryos (compare Fig. 6B, panel B and D). In addition, XA-1 is also expressed in the cement gland of Keller sandwiches (Fig. 6B, panel D). In this particular explant, two distinct cement glands have developed (only one is visible in panel D). In one Keller sandwich analyzed, XA-1 expression in cement gland tissue is localized to a band of cells around the explant (data not shown), similar to the expression of XAG-1 seen in Figure 6B, panel C.

These results suggest that in addition to patterning the neural plate, planar signals may also play a role in induction of the most anterior dorsal ectoderm, the cement gland and hatching gland. These organs develop in the same anteroposterior position in these explants relative to neural tissue, and show extensive morphological organization.

## Cement gland development in isolated dorsal ectoderm

Using molecular markers expressed in the cement gland, it has been possible to examine in more detail the anteroposterior patterning and induction of the ectoderm during gastrulation. The cement gland itself has been shown to possess anteroposterior polarity, in the expression of XA-1, which is confined to the posterior portion of the developing cement gland (see Fig. 1). Therefore, in addition to offering clues as to how the ectoderm is patterned overall, the cement gland itself provides an opportunity to look in detail at how anteroposterior patterning is established.

XA-1 expression is first detected in the cement gland around mid-neurula stages (st. 17, see Fig. 1I). Both XAG-1 and XCG-1 are expressed uniformly in the cement gland at this stage, but XA-1 is first detectable in a

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region of the cement gland that is contacted by the hatching gland.  Later in development, during tailbud and hatching gland stages (Fig. 1J, 1K), XA-1 expression expands but is still confined to the posterior portion of the cement gland.  These patterns of expression in whole embryos suggest that establishment of XA-1 expression in the cement gland may be mediated by planar signals within the dorsal ectoderm.  XA-1-positive presumptive hatching gland tissue may in turn signal adjacent cells in the posterior of the cement gland to express XA-1 as well.

A series of experiments were performed to determine which tissues and cell interactions are necessary for differentiation and patterning of the cement gland.  First, experiments were performed to investigate the possibility that planar signalling from more posterior ectoderm is responsible for XA-1 asymmetry in the cement gland.  Induced dorsal ectoderm, including tissue $50^o$ on either side of the dorsal midline but excluding ectoderm adjacent to the blastopore lip, was dissected from mid-gastrula stage embryos (st. 11.5, Fig. 7).  This tissue included prospective cement gland and neural plate ectoderm (Keller, 1975).   These explants were cultured in neutral saline until they reached late tailbud stage (st. 28) equivalent.  Explants elongate and develop axial structures such as cement and hatching glands that are easily identifiable under the dissecting microscope.  Explants from albino embryos were used for *in situ* hybridization studies to look at the expression of genes in the cement and hatching glands.

Dorsal ectoderm explants produce cement glands that are similar to those seen in whole embryos:  they are comprised of elongate cells and stick to forceps, suggesting that they secrete mucus like normal differentiated cement glands.  The only obvious morphological difference is that the

Figure 7. Diagrams of explants dissected at mid-gastrula stage (stage 11.5). Top diagram depicts an embryo seen from a dorsovegetal view; bottom embryo is a sagittal section of the same stage. Dorsal ectoderm explants (hatched region) corresponding to induced ectoderm from $50^{\circ}$ on either side of the midline were dissected. These dorsal ectoderm explants excluded tissue adjacent to the blastopore lip. Dorsal ectoderm + mesendoderm (stippled region) explants included the blastopore lip. All explants were allowed to develop in 1X MBS until control embryos reached tailbud stage (st. 28) equivalent. *In situ* hybridization of fixed explants and embryos are shown in Figure 8.

St. 11.5
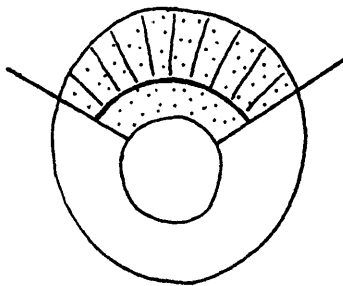
dorsovegetal view
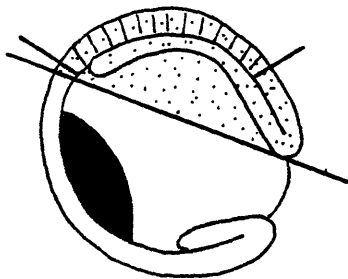
lateral view

dorsal ectoderm

or

dorsal ectoderm + mesendoderm

neutral saline

St. 28 equivalent

cement glands formed in the dorsal ectoderm explants are smaller than those in the whole embryo. In addition, the pattern of XAG-1 expression in the dorsal ectoderm explants is the same as seen in equivalent stage embryos (Fig. 8A and B). In all dorsal ectoderm explants analyzed (11), XAG-1 transcripts are distributed uniformly throughout the cement gland and are also detected in hatching gland cells in many of the explants (8/11). The cement gland-specific marker XCG-1 is also expressed exclusively in the cement gland in both whole embryos and dorsal ectoderm explants (Fig. 8D and E). Dorsal ectoderm explants display an extensive amount of hatching gland development, as seen by detection of UVS.2 (Fig. 8J and K). Hatching gland specific XA-1 transcripts are detected in most explants (15/18; Fig. 8H) where their pattern of expression is similar to that seen in whole embryos (Fig. 8G) and in UVS.2 stained explants (Fig. 8K). However, the localization of XA-1 in cement glands of dorsal ectoderm explants is strikingly different to that seen in whole embryos. In the majority of dorsal ectoderm explants analyzed (14/18), XA-1 RNA is no longer restricted to the posterior portion of the cement gland, but is expressed throughout it (compare Fig. 8G and H). Therefore, although cement gland development in dorsal ectoderm explants appears indistinguishable from normal cement glands based on morphology and the expression of XAG-1 and XCG-1 genes, they are not identical in terms of the restriction of XA-1 expression. Further, these results suggest that although planar signals from more posterior tissue may be necessary for expression of XA-1 in the cement gland, they are not responsible for the anteroposterior restriction of XA-1 RNA.

**Inhibitory signals from tissue ventral to the cement gland?**

Figure 8. Patterning of the cement gland in dorsal ectoderm and dorsal ectoderm + mesendoderm explants.
All panels show a view of cement gland and hatching gland tissue. Panels A, D, G, and J show are control tailbud embryos. Panels B, E, H, K are explants of dorsal ectoderm alone and panels C, G, I, L are explants of dorsal ectoderm + mesendoderm. All magnifications are 40X.

(A) XAG-1 expression in tailbud stage (st. 28) embryo. XAG-1 is expressed throughout the cement gland. (B, C) XAG-1 is expressed uniformly in the cement gland in explants of dorsal ectoderm (B) and in dorsal ectoderm + mesendoderm explants. Note the extensive head development in dorsal ectoderm + mesendoderm explants (C, F, I, L). A low level of XAG-1 is also detected in the hatching gland in both explants (B, C). (D, E, F) XCG-1 expression is cement gland specific in whole embryos (D) as well as in dorsal ectoderm explants (E) and dorsal ectoderm + mesendoderm explants (F). There is no detectable XCG-1 expression elsewhere in the embryo or in explants. (G) Expression of XA-1 is detected in the entire hatching gland and in a restricted region (posterior) of the cement gland in whole embryos. This posterior restriction is not seen in explants of dorsal ectoderm alone (H) where XA-1 is expressed throughout the developing cement gland. (I) A partial restoration of XA-1 asymmetry in the cement gland is seen in dorsal ectoderm + mesendoderm explants. XA-1 is also expressed in hatching gland in both explants (H, I). (J, K, L) UVS.2 is exclusively expressed in the hatching gland of embryos (J), dorsal ectoderm explants (K), and in explants of dorsal ectoderm + mesendoderm (L).

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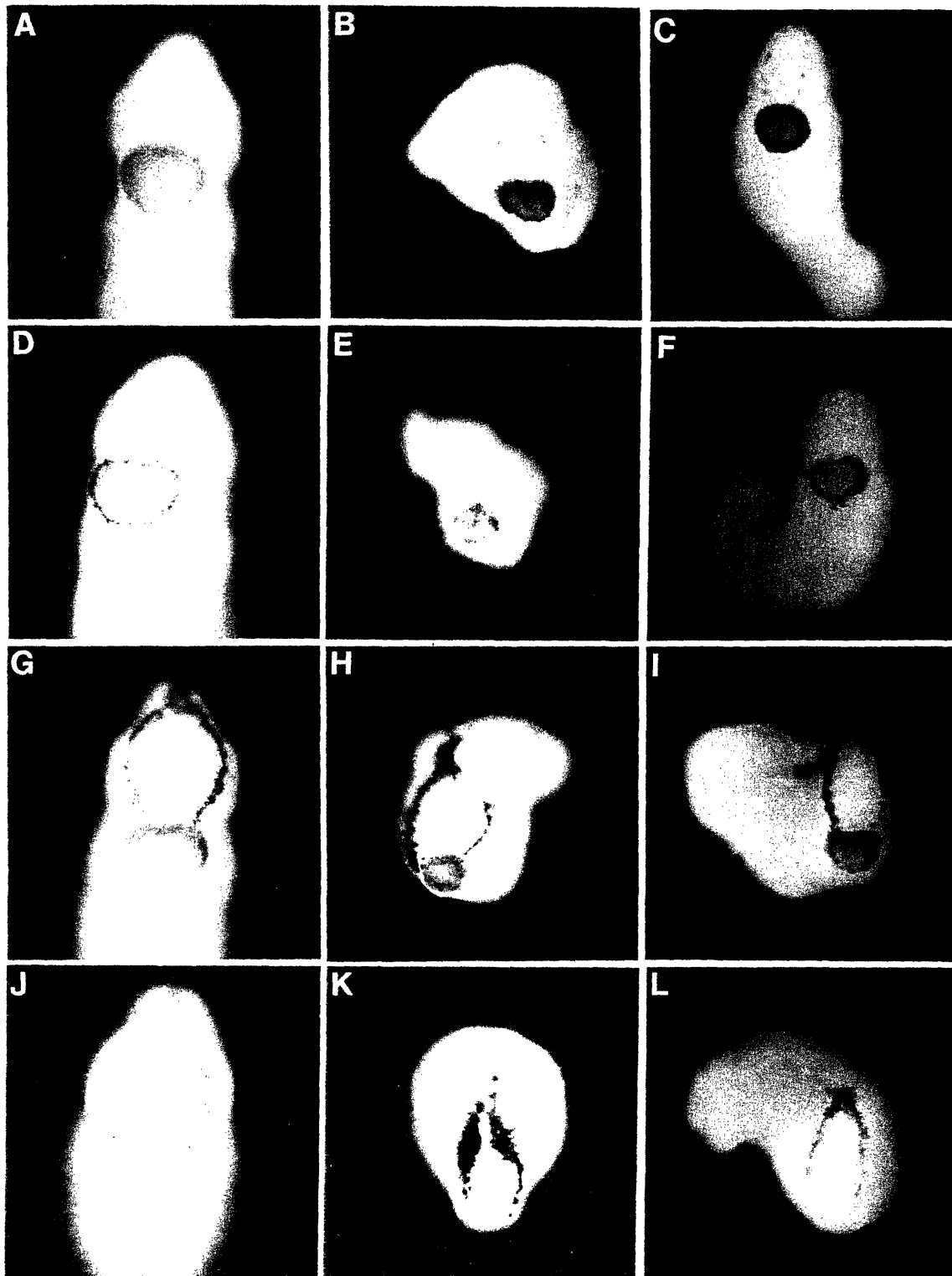
The dorsal ectoderm has aquired a great deal of anteroposterior patterning by mid-gastrula stages, as demonstrated by the extensive anterior development observed in dorsal ectoderm explants. Although isolated dorsal ectoderm from mid-gastrula stages can support cement gland differentiation, signalling within the ectoderm is not sufficient to account for the complete patterning of the cement gland. Following gastrulation, signalling from other tissue(s) besides the ectoderm may be required for anteroposterior patterning within the cement gland.

To address whether asymmetric expression of XA-1 in the cement gland requires tissue other than ectoderm, explants were prepared from mid-gastrula stage embryos. The tissue explanted is shown in Figure 7. Each explant contained dorsal ectoderm as well as underlying mesoderm and some deep endoderm. The dissected tissue included the dorsal lip as well as tissue 50$^o$ on either side of the marginal zone. Explants developed in neutral saline until control embryos reached st. 28, and displayed a great deal of dorsoanterior development. Well-formed heads with cement glands and eyes were easily identifiable.

Cement gland and hatching gland development in these explants was monitored by *in situ* hybridization, using the same panel of genes as in the previous set of experiments. The profile of XAG-1 expression is the same as seen in whole embryos and explants of dorsal ectoderm alone. In all dorsal ectoderm + mesendoderm explants analyzed (23) XAG-1 is expressed throughout the cement gland, and in about half of these explants (12/23) it is also expressed in a subset of cells in the hatching gland (Fig. 8C). The morphology of the cement and hatching glands in the explant vs. the whole embryo is virtually identical, although the head of the explant is smaller

than that of the embryo (compare Figs. 8A and C). XCG-1 expression is also indistinguishable in cement glands of explants vs. whole embryos (compare Figs. 8D and F). Hatching gland development can also be seen in explants probed with UVS.2 (Fig. 8L). XA-1 RNA is detected in both hatching gland and cement gland in 29 explants (Fig. 8I). The pattern of XA-1 expression in the hatching gland of dorsal ectoderm + mesendoderm explants is similar to that of UVS.2. This is consistent with the expression of the two genes in the hatching glands of whole embryos and dorsal ectoderm explants. However, the pattern of XA-1 expression in the cement gland of dorsal ectoderm + mesendoderm explants is different from that seen in dorsal ectoderm explants alone. In constrast to the uniform expression in the dorsal ectoderm explants, XA-1 RNA is not distributed throughout the cement gland in most of the dorsal ectoderm + mesendoderm explants analyzed (27/29). In these explants XA-1 expression is enhanced in the posterior region of the cement gland and is reduced or absent from the anterior portion, as it is in normal embryos (compare Figs. 8G and I).

These results indicate that signals arising from the dorsal or lateral mesendoderm may be responsible for the asymmetric distribution of XA-1 RNA in the developing cement gland. This signalling appears to inhibit the expression of XA-1 in the anterior half of the cement gland, since XA-1 expression is uniform in cement glands derived from explants of dorsal ectoderm alone. A potential source of inhibitory signal is the heart anlage, which is adjacent to the anterior edge of the developing cement gland.

## DISCUSSION

The experiments reported here investigated the induction and patterning of

the cement gland during *Xenopus laevis* embryogenesis. By using a set of genes expressed specifically in the developing cement gland, I have analyzed both the induction of the cement gland during gastrula stages and the subsequent cell-cell interactions necessary for patterning within the organ.

**Early induction of the cement gland: intraectodermal signalling?**

Induction of the cement gland marker, XAG-1, occurred in dorsal ectoderm isolated from gastrula stage embryos. Separation of the ectoderm into individual layers indicated that cells of the inner layer are initially specified and express XAG-1 early in gastrulation whereas cells in the outer layer are subsequently induced later in development. Moreover, since the tissue isolated early in gastrulation does not contribute to the mature cement gland but to part of the neural plate (Sive et. al., 1989), the induction of XAG-1 in the outer layer of the ectoderm does not occur until later in gastrulation when presumptive cement gland cells are induced.

Sive et. al. (1989) have proposed that the ectoderm is progressively patterned during gastrulation with the posterior ectoderm transiently induced as more anterior tissue. It is a possibility that XAG-1 induction occurs only in the inner layer of the ectoderm early in gastrulation because that tissue is to be respecified as neural tissue later in development. Induction of cement gland markers in the outer layer of the ectoderm may occur only in tissue fated to contribute to the developing cement gland. Drysdale and Elinson (1992) performed a series of experiments which involved transplantation of labelled inner or outer cells from uninduced ectoderm into early gastrula stage host embryos. These embryos were allowed to develop to tailbud stages and analyzed for the presence of labelled

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cells in the mature cement and hatching glands. They found that the majority of cells that contribute to both the cement gland and the hatching gland are derived from the outer layer of the ectoderm. The induction of both XAG-1 and XA-1 in the outer layer of anterior dorsal ectoderm is detected by late gastrula stages, and it is possible that the expression of these genes is maintained only in the outer layer of cells later in development when the cement and hatching glands are becoming morphologically distinct. Alternatively, it could be that the outer layer actively represses the expression of cement and hatching gland genes in the inner layer later in development.

How does signalling within the ectoderm occur? Two of several possible scenarios for how this induction takes place are 1) the underlying or adjacent mesoderm produces an inductive signal which first acts on the adjacent inner layer of the ectoderm early in gastrulation and by later gastrula stages has diffused through the inner layer and penetrates the outer layer, or 2) the inner layer of the ectoderm is first induced by the underlying mesoderm and then in turn the inner layer itself signals the adjacent outer layer of the ectoderm. These theories have been advanced by Grunz (1990) in studies of homeogenetic induction. He found that ectoderm from late gastrula embryos corresponding to presumptive neural plate could induce neural structures in competent early gastrula ectoderm. Moreover, he found that the inductive capacity of the inner layer alone was greater than both layers together. He proposed that this could be the case if the outer layer of the ectoderm has not been fully induced yet by either a primary signal arising from the underlying chordamesoderm, or homeogenetically by the inner layer of the ectoderm. Analysis of cement gland induction by the dorsal

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mesoderm has shown that at mid-gastrula stages the tissue with the greatest cement-gland inducing capacity is posterior to ectoderm fated to become cement gland (Sive et. al., 1989). Therefore, the vertical arrangement of underlying mesoderm directly signalling adjacent ectoderm is not sufficient to explain cement gland induction. It seems likely that both planar and vertical signalling play a role in the induction of cement gland markers in the inner and outer layers of the ectoderm.

**Planar signals induce cement gland**

It has been shown that dorsal mesoderm isolated from gastrula stage embryos can induce cement gland tissue in naive ectoderm, presumably by signals which pass vertically from the mesoderm to the ectoderm in these recombinants (Sive et. al., 1989). To investigate whether planar signals arising from the dorsal mesoderm are sufficient for the differentiation of cement gland tissue in competent ectoderm, XAG-1 and XA-1 expression was analyzed in Keller sandwiches (Keller and Danilchik, 1988). Signalling from the mesoderm to the ectoderm can occur only in a planar fashion, as involution of the mesoderm under the ectoderm is prevented. Both XAG-1 and XA-1 are expressed in these explants. XAG-1 positive cells often form a ring around the explant and presumably correspond to cement gland cells, based on cellular morphology and the secretion of mucus. XA-1 expression is detected in a population of cells which contribute to both cement and hatching gland tissue. Moreover, the organization of the hatching gland is remarkably similar to that seen in the whole embryo, with two parallel lines of cells extending from the cement gland posteriorly into the anterior neural plate region. Additionally, the expression of XAG-1 and XA-1 in Keller

sandwiches is in an appropriate anteroposterior position relative to expression of the neural marker, *en-2*. Taken together, these results confirm that planar signals alone can induce the formation of cement and hatching gland tissues. Since these structures demarcate the cephalic end of the embryo, they correspond to the most anterior tissue observed to develop in Keller sandwiches.

These results extend previous studies which showed that inductive signals passing planarly from the dorsal mesoderm to the ectoderm can account for extensive patterning of neural tissue in both exogastrulae (Kintner and Melton, 1987; Dixon and Kintner, 1989; Ruiz i Altaba, 1992) and Keller explants (Keller and Danilchik, 1988; Doniach et. al., 1992). However, planar induction alone cannot account for complete development of the head, and there is evidence to support the theory that both planar and vertical from the mesoderm may act synergistically to mediate induction and development of the dorsal ectoderm (Dixon and Kintner, 1989; Ruiz i Altaba, 1992). The cement gland tissue that develops in Keller sandwiches is not morphologically identical to that in the whole embryo, therefore it is possible that planar induction contributes to only one aspect of cement gland induction. Additional vertical signalling may also be required for normal development of the cement gland.

Several studies suggest that another source of planar signals, those arising from neural tissue, may also play a role in induction of the cement gland. The cement gland develops from a mesoderm-free region of the ectoderm that is anterior to the presumptive neural plate. As stated previously, Sive et. al. (1989) have shown that in mid-gastrula stage embryos the region of mesoderm capable of inducing the highest level of cement

gland markers is far posterior to ectoderm fated to become cement gland tissue. Therefore it is possible that the anterior neural plate, which is adjacent to the presumptive cement gland ectoderm, may contribute to the development of the cement gland by means of planar induction. Itoh and Kubota (1991) found that isolated anterior plate, when combined with competent ectoderm, could induce expression of a neural-specific antigen in the responding ectoderm. Cement glands derived from the competent ectoderm were also observed, suggesting that the anterior neural plate alone is capable of inducing cement gland tissue. Drysdale and Elinson (1993) subdivided the neural plate into different regions and found that isolated anterior but not posterior neural plate is capable of inducing cement gland differentiation in competent ectoderm. Subsequent dissection of the anterior neural plate showed that the rostral edge was a better inducer of cement gland than the lateral edge, which is consistent with the theory that the cement gland inducing capacity is greatest in the portion of the neural plate adjacent to the presumptive cement gland tissue. Induction of cement gland in Keller explants may occur in a similar fashion, since the anteroposterior position of tissues is comparable to that in a normal embryo. What is not known is whether these inductive signals are produced by the neural plate itself or if they originate from the dorsal mesoderm and then diffuse through the ectoderm until they reach the presumptive cement gland region, which is competent to respond to these signals. A candidate for such a diffusible inducer is noggin, a novel secreted protein that is expressed in the dorsal mesoderm in the region called Spemann's organizer (Smith and Harland, 1992). Purified human noggin protein can induce the expression of cement gland (XAG-1) and anterior neural markers, suggesting that it may

play a role in induction of both of these tissues during normal development (Lamb et. al., 1993).

**Anteroposterior patterning of the cement gland occurs during neurula stages**

The developing cement gland has anteroposterior polarity as shown by the restricted expression of XA-1. This is a surprising result, given that the cement gland is thought to be comprised of a homogeneous group of cells. The expression of XA-1 in the cement gland does not occur until neurula stages, suggesting that the restriction of XA-1 in this tissue is mediated by events that are distinct from early induction of the cement gland during gastrula stages.

In vitro induction of competent ectoderm with NH4Cl results in the formation of cement gland tissue, identifiable morphologically and by the expression of cement gland specific markers, XAG-1 and XCG-1. XA-1 is also expressed in these explants at a similar level as in normal cement glands. However, the organization of the cement gland tissue induced by NH4Cl is different from normal cement glands as XA-1 positive cells are not spatially restricted. This suggests that cement gland induction by NH4Cl is incomplete and cannot account for the anteroposterior patterning of the cement gland. One explanation is that treatment of ectoderm with NH4Cl can mimic the early stages of cement gland induction, but the cell-cell interactions necessary for anteroposterior patterning of the cement gland later in development are not present in these explants.

Induced dorsal ectoderm isolated from mid-gastrula stage embryos can support cement gland and hatching gland development. These structures become morphologically distinct during development and also express the

same panel of markers as normal embryos. Both XAG-1 and XCG-1 transcripts are distributed uniformly throughout the cement glands of these explants, as in the whole embryo. Hatching gland development is confirmed by UVS.2 expression, which is specific to this tissue in both explants and embryos. One striking difference in cement gland differentiation is that XA-1 is no longer localized to the posterior region of the cement gland in dorsal ectoderm explants, but is expressed throughout it. These results suggest that the dorsal ectoderm alone, when isolated during mid-gastrula stages, is not capable of establishing anteroposterior patterning within the cement gland. However, it can account for expression of XA-1 in the cement gland which, as suggested in studies of planar induction, may be the result of signalling from more posterior dorsal ectoderm included in the explants.

XA-1 is first detectable in the posterior region of the developing cement gland during mid-neurula stages. The expression of XA-1 throughout the cement gland in dorsal ectoderm explants suggests that an inhibitory signal arising from tissue other than the dorsal ectoderm may be required for the spatial restriction of XA-1. Explants from mid-gastrula stages containing both dorsal ectoderm and underlying mesendoderm display a great deal of dorsoanterior development. Cement and hatching gland formation in these explants is very similar to whole embryos, as determined by the expression of XAG-1, XCG-1 and UVS.2. In addition, the asymmetric distribution of XA-1 is partially restored in these explants, with levels of XA-1 RNA higher in the posterior region of the cement gland. This asymmetry is presumably due to signalling from some portion of the dorsal or lateral mesendoderm, which was not present in the dorsal ectoderm explants.

If the lack of XA-1 expression in the anterior portion of the cement

gland is due to a repression of XA-1 in this region, then the likely source of this inhibitory signal is the tissue that lies just ventral to the presumptive cement gland. In the developing *Xenopus* embryo this tissue corresponds to the heart anlage. In early gastrula stage embryos the heart mesoderm is derived from two regions of the deep mesoderm lateral to the blastopore lip (Keller, 1976). During gastrulation, the prospective heart mesoderm moves ahead of the chordamesoderm (Gerhart and Keller, 1986). It subsequently migrates laterally and ventrally during neurula stages until it fuses at the midline, just below the presumptive cement gland (reviewed in Jacobson and Sater, 1988). Sater and Jacobson (1990) have shown that both the dorsal blastopore lip and the deep mesoderm from $30^O$ to $45^O$ on either side of the dorsal midline are necessary for heart formation.

The dorsal ectoderm + mesendoderm explants dissected in this current study include both the blastopore lip and tissue $50^O$ on either side of the dorsal midline. Therefore, these explants contain presumptive heart mesoderm, although explants were harvested during tailbud stages before beating hearts could be observed so heart development was not unequivocally identified. However, Jacobson and Sater (1989) have shown that the specification of heart mesoderm in *Xenopus* occurs during gastrulation. They have also shown that explants from mid-gastrula stage embryos (St. 11.5) which include both the blastopore lip and the dorsal region $45^O$ on either side of the midline undergo heart formation in all cases (Sater and Jacobson, 1990). The inclusion of presumptive heart mesoderm is consistent with the observation that asymmetric XA-1 expression is restored in the ectoderm + mesendoderm explants. Moreover, the ventral fusion of the heart anlage at the midline is during the same time in development

when XA-1 expression is first detected in the cement gland. Therefore, the heart anlage is in the right place at the right time to play a role in the anteroposterior patterning of the cement gland by inhibiting XA-1 expression in the portion of the cement gland adjacent to it. Additional experiments will need to be performed to test whether the heart anlage directly influences cement gland patterning.

**Induction of the cement gland: a multi-step process**

The results of these and previous studies indicate that the anteroposterior patterning of the dorsal ectoderm is a very complex process, requiring multiple inductive events. Molecular analysis of cement gland induction has been facilitated by the availability of genes that are specifically expressed in the developing tissue, both early in development when the anteroposterior axis is established during gastrulation, and later during differentiation of the organ.
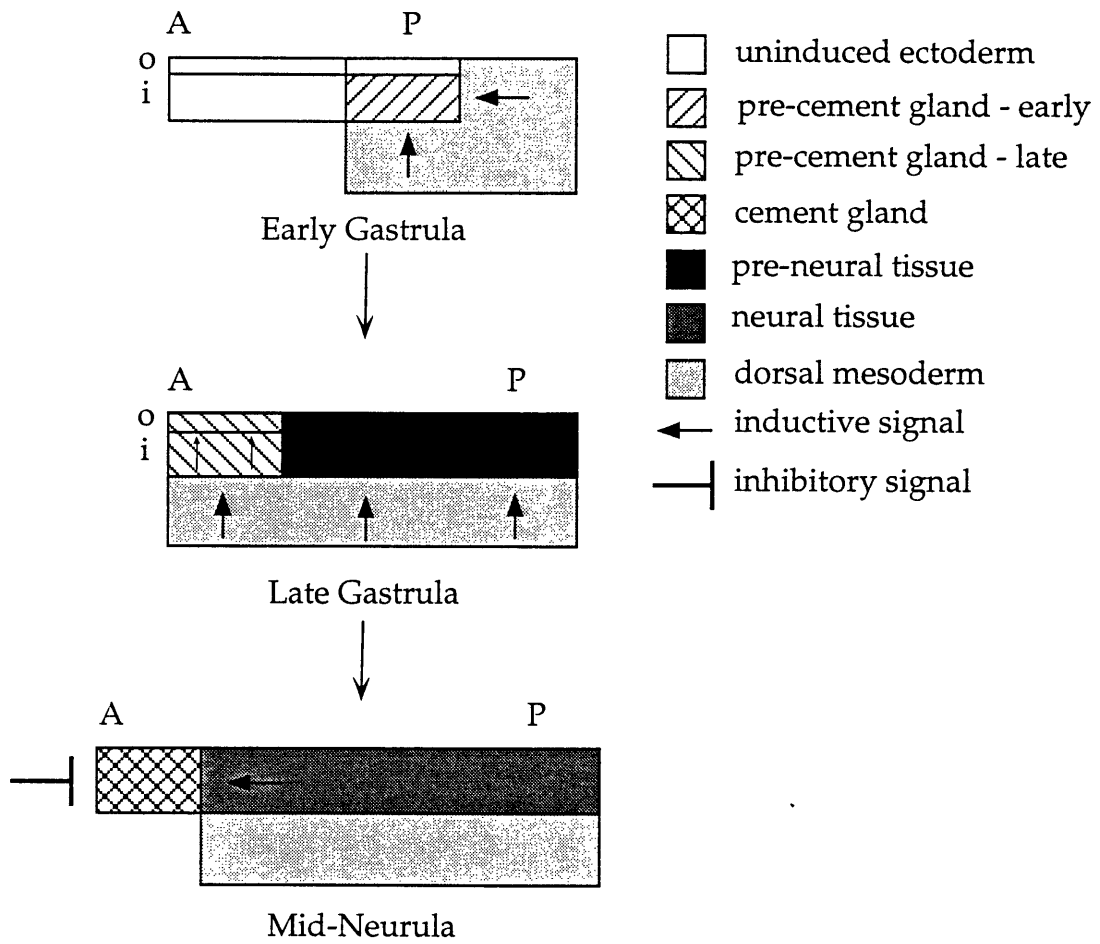
Despite the simple organization of the cement gland, it is clear that it's induction and organization require multiple interactive events which occur at different times during development. A model outlining some of the proposed steps in cement gland induction and patterning is shown in Figure 9. Key features of the stepwise determination of the cement gland include 1) the restriction of the presumptive tissue to the anterior region of the dorsal ectoderm during gastrulation, a process which appears to involve planar and appositional signalling and respecification of more posterior ectoderm, and 2) differentiation and patterning of the cement gland during neurula stages which seems to be mediated by both inductive and inhibitory interactions with neighboring tissues.

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Figure 9. Multi-step induction and patterning of the cement gland
A = anterior, P=posterior, i=inner layer of the ectoderm, o=outer layer of the
ectoderm

Early Gastrula: The initial inductive events occur early in gastrulation and
are mediated by the dorsal mesoderm which is capable of instructing the
ectoderm to form cement gland in either a planar or appositional fashion.
The inner layer of the ectoderm is first specified as pre-cement gland tissue.

Late Gastrula: The initial induction of pre-cement gland is transient, and
more posterior ectoderm in respecified as pre-neural tissue later in
gastrulation. At this time both the inner and outer layers of the ectoderm are
specified as cement gland tissue, possibly due to vertical induction of the
outer layer by the inner layer.

Mid-Neurula: Cement gland and neural tissue are determined during
neurula stages. Planar signals arising from more posterior neural plate may
influence both the position and determination of the presumptive cement
gland, and ventral inhibitory signals may be involved in susequent
patterning of the developing cement gland.

Early Gastrula

Late Gastrula

Mid-Neurula

uninduced ectoderm
pre-cement gland - early
pre-cement gland - late
cement gland
pre-neural tissue
neural tissue
dorsal mesoderm
inductive signal
inhibitory signal

Developmental processes similar to those observed in cement gland formation have been shown to occur in the development of the lens, which is also derived from anterior dorsal ectoderm (reviewed in Grainger, 1992). During gastrula and neurula stages, lens-inducing signals are thought to be transmitted through the plane of the ectoderm from the presumptive neural plate (Henry and Grainger, 1990). Additionally, induction of lens tissue is thought to be enhanced by tissues underlying the lens area. Therefore, planar and appositional inductions appear to play a role in the initial induction of both lens and cement gland tissue. The determination and differentiation of lens tissue later in development is influenced by other tissues including the optic vesicle, which may determine the precise location of the lens in the head (Grainger et. al., 1988), and migrating neural crest cells, which may inhibit lens formation in other tissues (Henry and Grainger, 1987; Jacobson and Sater, 1988). Both inductive and suppressive interactions later in development seem to play a role in the differentiation of both cement gland and lens tissue. The similarity of steps involved in the formation of the lens and the cement gland suggests that the induction and differentiation of these tissues may reflect developmental processes that are shared by the anterior dorsal ectoderm.

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