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Positive and negative signals modulate formation of the *Xenopus* cement gland

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SUMMARY

The cement gland is a simple secretory organ that marks the anterior-most dorsal ectoderm in *Xenopus* embryos. In this study, we examine the timing of cement gland induction and the cell interactions that contribute to cement gland formation. Firstly, we show that the outer ectodermal layer, from which the cement gland arises, becomes specified as cement gland by mid-gastrula. Curiously, at early gastrula, the inner layer of the dorsal ectoderm, which does not contribute to the mature cement gland, is strongly and transiently specified as cement gland. Secondly, we show that the mid-gastrula dorsoanterior yolky endoderm, which comes to underlie the cement gland primordium, is a potent inducer of cement gland formation and patterning. The cement gland itself has an anteroposterior pattern, with the gene *XA* expressed only posteriorly. Dorsoanterior yolky endoderm greatly enhances formation of large, patterned cement glands in partially induced anterodorsal ectoderm, but is unable to induce cement gland in naive animal caps. Neural tissue is induced less frequently than cement gland by the dorsoanterior yolky endoderm, suggesting that the endoderm induces cement gland directly. Thirdly, we demonstrate that the ventral ectoderm adjacent to the cement gland attenuates cement gland differentiation late during gastrulation. The more distant ventral mesendoderm is also a potent inhibitor of cement gland formation. These are the first data showing that normal ventral tissues can inhibit cement gland differentiation and suggest that cement gland size and position may be partly regulated by negative signals. Previous work has shown that cement gland can be induced by neural plate and by dorsal mesoderm. Together, these data suggest that cement gland induction is a complex process regulated by multiple positive and negative cell interactions.

Key words: *Xenopus*, cement gland, induction, ventral ectoderm, endoderm

INTRODUCTION

The cement gland is a simple mucus-secreting organ positioned at the front of the *Xenopus* embryo. It attaches the newly hatched embryo to a support before the hatchling can swim well or feed. Together with sensory neurites that innervate it, the cement gland mediates a 'stopping response' when the tadpole is safely attached by its glue (Boothby and Roberts, 1992). In addition, the cement gland acts as an excellent positional marker, for several reasons. Firstly, it is a highly visible indicator of the extreme anterior dorsal ectoderm. Secondly, recent data suggest that some of the molecular signals involved in neural induction may also be involved in cement gland formation (Ekker et al., 1995; Hemmati-Brivanlou et al., 1994; Lamb et al., 1993; Sasai et al., 1995). However, cement gland differentiates many hours before the first neurons do (Hartenstein, 1989; Nieuwkoop and Faber, 1967; Sive and Bradley, 1996) suggesting that cement gland induction may be simpler to analyze than neural induction. Thirdly, since the cement gland forms at the boundary between dorsal and ventral ectoderm, it may be an indicator of cell interactions that take place at this boundary (Sive and Bradley, 1996).

Cement gland induction has clearly occurred by late gastrula, when the cement gland marker genes *XCG* and *XAG* are first expressed throughout the cement gland primordium (Sive and Bradley, 1996). From early neurula, the gene *XA* is expressed asymmetrically across the cement gland, only in the posterior half (Hemmati-Brivanlou et al., 1990; Sive and Bradley, 1996). We previously employed specification assays to show that dorsal ectoderm isolated from early gastrulae and cultured in vitro, goes on to express low levels of cement gland markers. As gastrulation proceeds cement gland specification strengthens, as judged by the level of marker gene expression observed in explants (Sive et al., 1989). The absolute position of specified cement gland in the dorsal ectoderm also moves during gastrulation, such that early, cement gland is specified in the presumptive neural plate. Later, specified cement gland is present more anteriorly, in or near the real cement gland primordium (Sive et al., 1989).

The embryonic ectoderm is composed of two layers, an outer monolayer (also called the 'epithelial' layer) and an inner layer several cells thick (also called the 'sensorial' layer) (Nieuwkoop and Faber, 1967). The cement gland forms from the outer ectodermal layer, as does the more posterior hatching gland and some neurons (Drysdale and Elinson, 1992; Hartenstein, 1989), while the inner layer gives rise to the bulk of the

neurectoderm (Hartenstein, 1989). The timing of cement gland induction in the outer ectodermal layer has not been determined.

We and others (Drysdale and Elinson, 1993; Sive et al., 1989; Yamada, 1938) previously showed that the dorsal mesoderm, particularly the presumptive notochord, is a potent inducer of cement gland. However, the notochord lies so far posterior to the cement gland primordium that is it unlikely to directly induce cement gland in the whole embryo. Early neurula stage neural plate is also a strong cement gland inducer (Drysdale and Ellinson, 1993; Yamada, 1938) and since this tissue directly abuts the cement gland primordium, it may be one of the tissues that normally contributes to cement gland formation. Two other tissues, the dorsoanterior yolky endoderm and the ventral ectoderm, lie adjacent to the cement gland suggesting that these could normally regulate cement gland formation. The cement-gland-inducing capacity of anterior endoderm has not been examined in detail (Yamada, 1938; Sive et al., 1989), while the activity of the ventral ectoderm has not been analysed previously.

In this report, we analyse the timing of cement gland induction in the outer ectodermal layer. We show that the dorsoanterior yolky endoderm is a potent cement gland inducer, and demonstrate that ventral ectoderm and ventral mesendoderm suppress cement gland differentiation in explants. These data suggest that cement gland induction is complex, involving both positive and negative signals.

MATERIALS AND METHODS

Embryos and dissections

Albino embryos were collected and dejellied, according to Sive and Cheng (1991). Cleavage stage embryos were stained with Nile blue sulphate (0.1 mg/ml in 0.1× MBS) in order to accurately determine the position of involuted and non-involuted tissues during gastrulation. Gastrula stage embryos were dissected on 1% agarose in 0.5× MBS using an eyebrow knife, as described in each figure. Explants and whole embryo controls were cultured in 0.5× MBS until collection at tailbud stages 24-27. Staging was according to Nieuwkoop and Faber (1967).

Whole-mount in situ hybridization

Double- and single-labeling whole-mount in situ hybridization was carried out on whole embryos and explants following a protocol which is adapted from the protocol of Harland (1991) and Lamb et al. (1993). Unless otherwise specified, all washes were for 5 minutes at room temperature, shaking horizontally in small mesh baskets (Costar) placed in 12-well tissue culture plates. Antisense RNA probes were made using digoxygenin-UTP and fluorescein-UTP (for *XCG* in the double-labeling protocol) according to the manufacturer's (Boehringer) instructions.

Probe hybridization and removal: Whole embryos or explants were treated as described in Harland (1991) except for the proteinase K treatment, which was for 5 minutes, at 2 μg enzyme/ml. The samples were incubated overnight at 60°C in hybridization mix (Harland, 1991) including 0.2 μg/ml digoxygenin-UTP-labeled probe. 0.2 μg/ml fluorescein-UTP-labeled probe was also included for doubles. Post-hybridization, a 10 minute wash in 50% PTw/50% formamide at 60°C,was followed by the SSC washes and RNAase treatment as described in Harland (1991).

Antibody treatment: Samples were pretreated with two washes in 100 mM maleic acid, 150 mM NaCl, pH 7.5 (MAB), 30 minutes in MAB including 2% Boehringer Mannheim blocking reagent (2% BMB) and then 1 hour in 20% heat-treated lamb serum in 2% BMB (LSBM). Samples were incubated in either anti-digoxygenin (singles), or anti-fluorescein (doubles), alkaline phosphatase linked antibody (Boehringer). The samples were incubated for 5 hours at room temperature, or at 4°C overnight with a 1:2000 dilution of antibody in LSBM. Excess antibody was removed with five 1 hour, room temperature MAB washes (one could be at 4°C overnight) followed by two washes in alkaline phosphatase (AP) buffer pH 9.5 (Harland 1991). The blue color reaction was carried out in AP buffer, pH 9.5 including 10% polyvinyl alcohol (Aldrich) with 4.5 μl NBT and 3.5 μl BCIP (Promega) per ml.

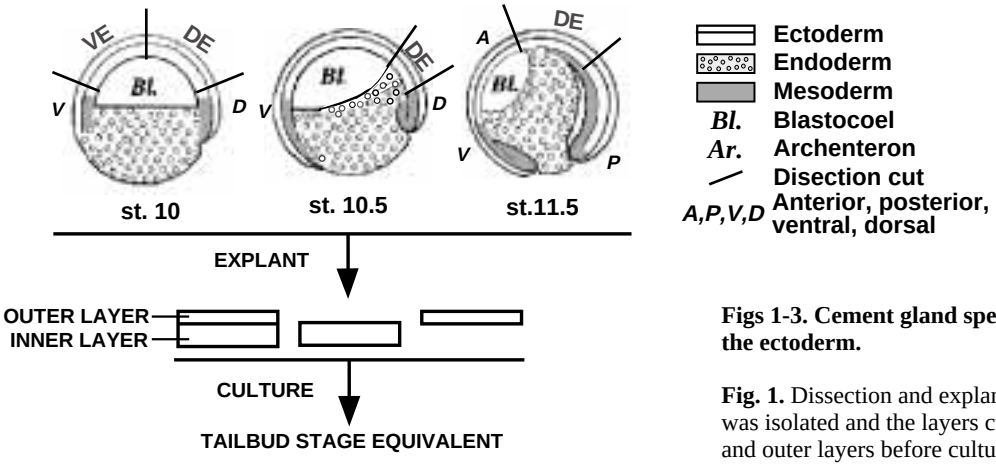
For double labeling: The first antibody was acid inactivated in 0.1 M glycine-HCl, pH 2.2/0.1% Tween-20, 40 minutes (Hauptmann and Gerster, 1994). This was followed by four washes in MAB (10 minutes each) and then 30 minutes in LSBM. The second antibody step was as above using anti-digoxygenin antibody. Two washes in 0.1 M Tris-HCl pH 8.2/0.1% Tween-20 were followed by the red color reaction, in one Fast Red tablet/ 2 ml of 0.1 M Tris-HCl pH 8.2/0.1% Tween-20 buffer (Boehringer).

RESULTS

Cement gland specification switches between ectodermal layers during gastrulation

Since the cement gland arises from the outer layer of the embryonic ectoderm, we wanted to determine when this layer became specified to differentiate as cement gland. We therefore explanted dorsal ectoderm at different times during gastrulation, and cultured both ectodermal layers together, or as separate inner and outer layers, until control embryos reached tailbud (stage 26) (Fig. 1). Cement gland differentiation was assayed by in situ hybridization for expression of *XCG* or *XAG* (not shown), with similar results. These data are documented in Fig. 2 and summarized in Fig. 3. We first tested very early gastrula (stage 10) ectoderm ('animal cap') that is not yet underlain by the dorsal mesoderm. Confirming previous results (Sive et al., 1989), *XCG* expression was not observed after culture of both intact ectodermal layers (Fig. 2a). However, after culture of the isolated inner ectodermal layer, we found that *XCG* transcripts were detected in multiple discrete patches in 100% of explants (Fig. 2b). Outer ectoderm isolated from early gastrula did not go on to express *XCG* (Fig. 2c). Since dorsal ectoderm is more 'predisposed' to form dorsal derivatives (Otte and Moon, 1992; Sharpe et al., 1987; Sokol and Melton, 1991) we tested both dorsal and ventral animal caps in this assay. The results shown in Fig. 2a-c were obtained from ventral caps; dorsal caps behaved in the same way (data not shown). Differentiation of the inner layer as cement gland did not appear to result from exposing these cells to the culture solution. Intact animal caps were kept open in two ways: (1) under a coverslip in 1× MBS, or (2) by culture in L-15 medium that prevents caps closing (Asashima and Grunz, 1983). In both cases, the inner layer remained exposed to the medium, but cement gland did not form (Asashima and Grunz, 1983 and not shown).

Slightly later during gastrulation, at stage 10.5, we repeated the specification assays with dorsal ectoderm that was underlain by involuting mesendoderm (Fig. 1). At this time, intact dorsal ectoderm was weakly specified as cement gland since 22% of explants went on to express *XCG* (Fig. 2d). The inner ectodermal layer was still specified as cement gland as 63% of isolated inner layers gave rise to *XCG*-expressing foci



Figs 1-3. Cement gland specification changes within the layers of the ectoderm.

Fig. 1. Dissection and explant culture scheme. Gastrula ectoderm was isolated and the layers cultured together, or separated into inner and outer layers before culture to tailbud stage equivalent.

(Fig. 2e). None of the outer ectodermal explants went on to express *XCG* (Fig. 2f).

By stage 11.5 (mid-gastrula) intact dorsal ectoderm (Fig. 1) was generally specified as cement gland since 60% of explants went on to express *XCG* in single large patches (Fig. 2g), similar to the cement glands of control embryos. Cement gland specification in the inner layer of mid-gastrula dorsal ectoderm had greatly declined relative to earlier stages, with only 14% of explants going on to express *XCG* (Fig. 2h). Conversely, the outer ectodermal layer had become specified as cement gland since 72% of outer layer explants expressed *XCG* in single patches (Fig. 2i).

The finding that early gastrula inner layer went on to express cement gland markers prompted us to ask whether the inner layer was specified for other lineages that would arise from the outer layer of the ectoderm in the whole embryo. Stage 10

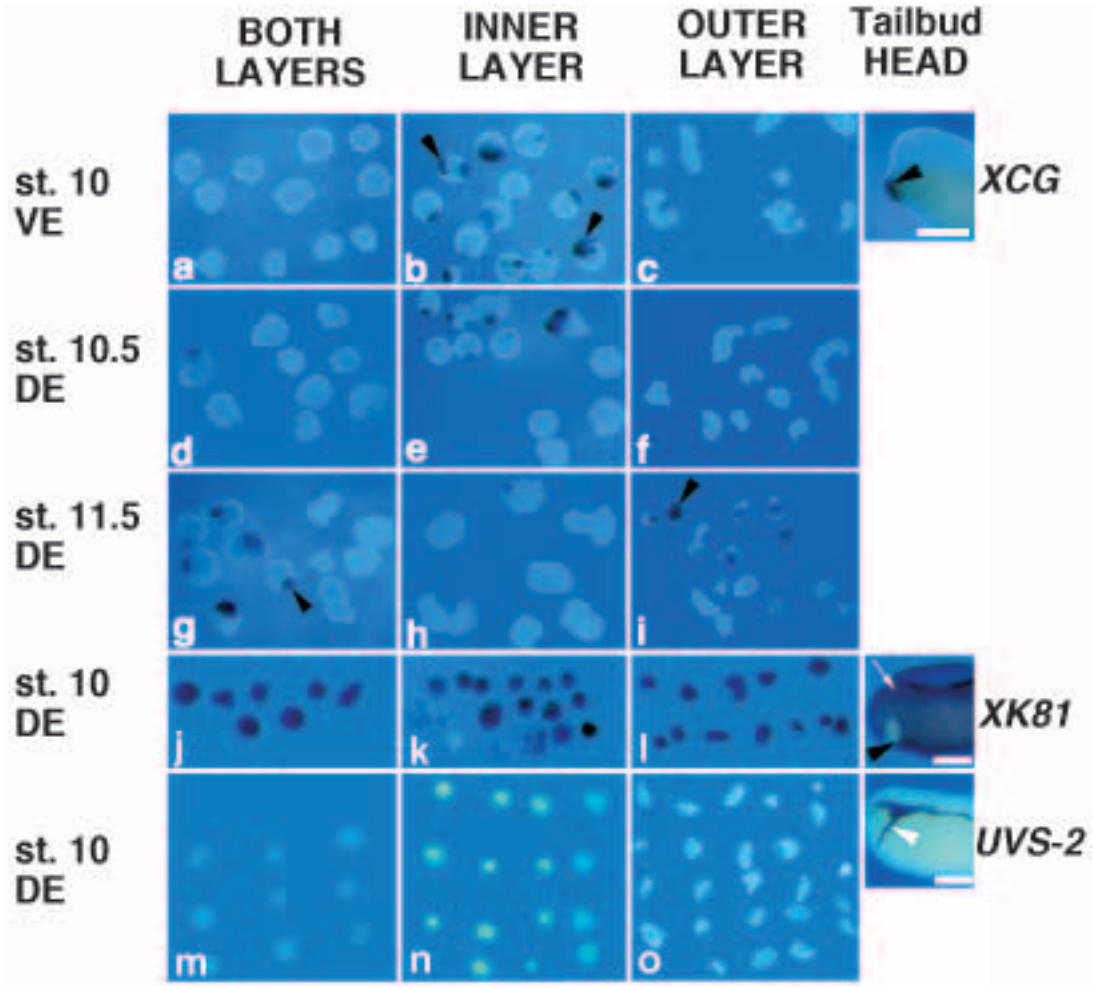


Fig. 2. In situ hybridization of cultured explants and tailbud stage control embryos. Antisense RNA probes were digoxygenin-UTP-labeled (dark blue). (a-c) Stage 10 ventral animal caps analyzed for *XCG* expression; (d-f) stage 10.5 dorsal ectoderm, underlain by mesendoderm and analyzed for *XCG* expression; (g-i) stage 11.5 dorsal ectoderm analyzed for *XCG* expression; (j-l) stage 10 animal caps analyzed for *XK81* expression; (m-o) stage 10 animal caps analyzed for *UVS-2* expression. Control embryos, photographed at the same magnification as explants, are shown in the right column. Black arrowheads show examples of cement gland staining, white arrowhead shows hatching gland and white arrow shows *XK81* staining. VE, ventral ectoderm; DE, dorsal ectoderm. The fourth column shows tailbud stage control embryos. Scale bar, 200 μm.

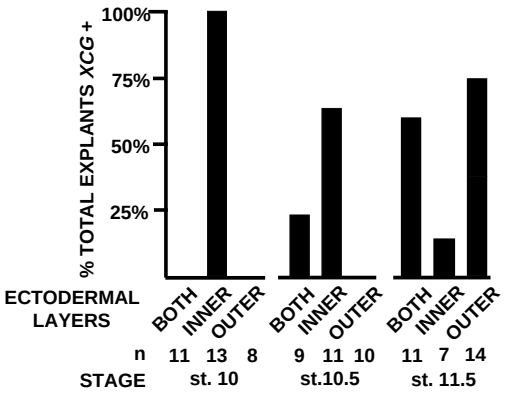


Fig. 3. Collated data analyzing the percentage of CG-positive explants cultured whole or as separate inner and outer layers relative to the total number of explants. The total number of explants examined is shown under each column. Similar results have been obtained for expression of another cement gland marker, *XAG* (not shown).

animal caps were cultured intact or as separate layers and later analyzed for expression of *XK81*, an epidermal cytokeratin gene (Sargent et al., 1986) or for *UVS-2* (Sato and Sargent, 1990) that is expressed in the hatching gland, which lies just posterior to the cement gland. As shown in Fig. 2j-l, in 100% of explants, intact ectoderm, as well as isolated inner and outer layers went on to express high levels of *XK81*. Expression of this gene in the inner layer was much patchier than expression in intact ectoderm or outer layer. The specification of epidermis in both ectodermal layers parallels results in intact caps where both layers express an epidermal antigen (Jones and Woodland, 1986). The hatching gland marker, *UVS2*, was not detected in cultured animal caps, whether they comprised both layers, the inner layer or the outer layer (Fig. 2m-o), indicating that specification of this gene was distinct from that of the cement gland (Drysdale and Elinson, 1993).

Together, these data showed that the outer layer of the ectoderm did not become specified as cement gland until the middle of gastrulation, whereas the inner layer, which does not contribute to the final cement gland, was transiently specified as cement gland by the onset of gastrulation.

The dorsoanterior yolky endoderm acts in conjunction with other signals to induce cement gland

By the end of gastrulation, the cement gland primordium is underlain by the dorsoanterior yolky endoderm (DAYE), part of the vegetal endoderm that lies anterior to the prechordal plate and that will eventually will give rise to the foregut (not shown and Nieuwkoop and Faber, 1967). We previously showed that anterior mesendoderm, including the DAYE, was not able to induce cement gland gene expression in early gastrula animal caps (Sive et al., 1989). However, we decided to examine the inducing activity of this tissue more thoroughly, asking whether the DAYE could induce cement gland formation and patterning at various points during gastrulation.

Cement gland patterning is shown in Fig. 4. In situ hybridization analysis revealed that *XCG* RNA (red) was expressed throughout the mature cement gland, whereas *XA* RNA (blue) transcripts were detected only in the cells of the posterior

Figs 4-8. Mid-gastrula dorsoanterior yolky endoderm induces patterned cement glands in anterior dorsal ectoderm.

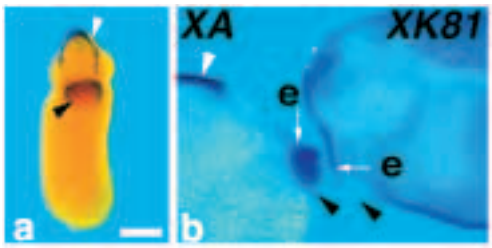


Fig. 4. Cement gland patterning. Double label in situ hybridization was carried out on stage 26 control embryos using digoxygenin-labeled *XCG* (in red) and fluorescein-labeled *XA* (in dark blue) antisense RNA probes (a). Single in situ hybridization was carried out on stage 26 embryos using digoxygenin-labeled *XA* and *XK81* probes (b). (a) Anterior view of a stage 26 embryo showing *XCG* transcripts in red, *XA* transcripts in blue. (b) Medial longitudinal sections of the heads of *XA*-(left) and *XK81*- (right) stained embryos. Black arrowheads indicate cement gland; white arrowhead indicates hatching gland; small white arrows indicate the epidermis, e, that surrounds the cement gland. Scale bar in a, 200 μm; embryos in b are at twice that magnification.

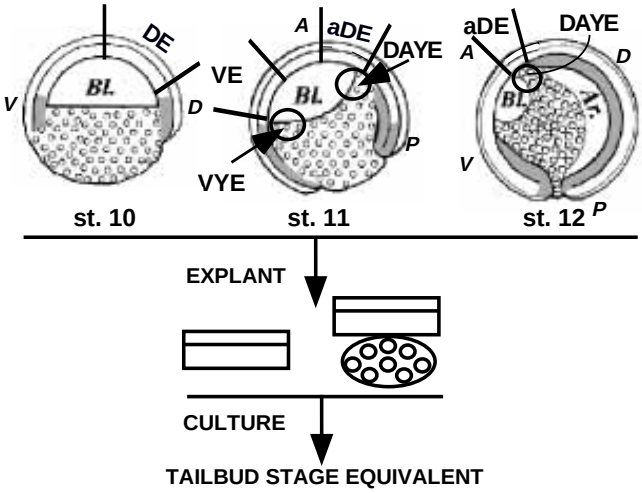


Fig. 5. Dissection and explant/conjugation culture scheme. Gastrula stage dorsal ectoderm (DE), anterior dorsal ectoderm (aDE), dorsoanterior yolky endoderm (DAYE) or ventral endoderm (VYE) was isolated and cultured either as ectoderm alone or as ectoderm/endoderm conjugates until tailbud stage equivalent. Same stage conjugates were made either by dissecting the ectoderm and endoderm separately and then putting them together or by dissecting the aDE in conjunction with the subjacent DAYE. Results were the same in either case. See Fig. 1 for key to tissues and abbreviations.

cement gland, and in the hatching gland which lies adjacent to the posterior cement gland (Fig. 4a; Hemmati-Brivanlou et al., 1990; Sive and Bradley, 1996). We define 'anterior' and 'posterior' in the cement gland based on the position of tissues during gastrula and neurula stages, where the posterior of the cement gland abuts the presumptive neural plate and the anterior abuts the archenteron. Later, as the head of the embryo curls down, 'anterior' is more ventral and 'posterior' cement gland is more dorsal, but the original 'anterior' and 'posterior' designations are still anatomically correct. Comparison of the

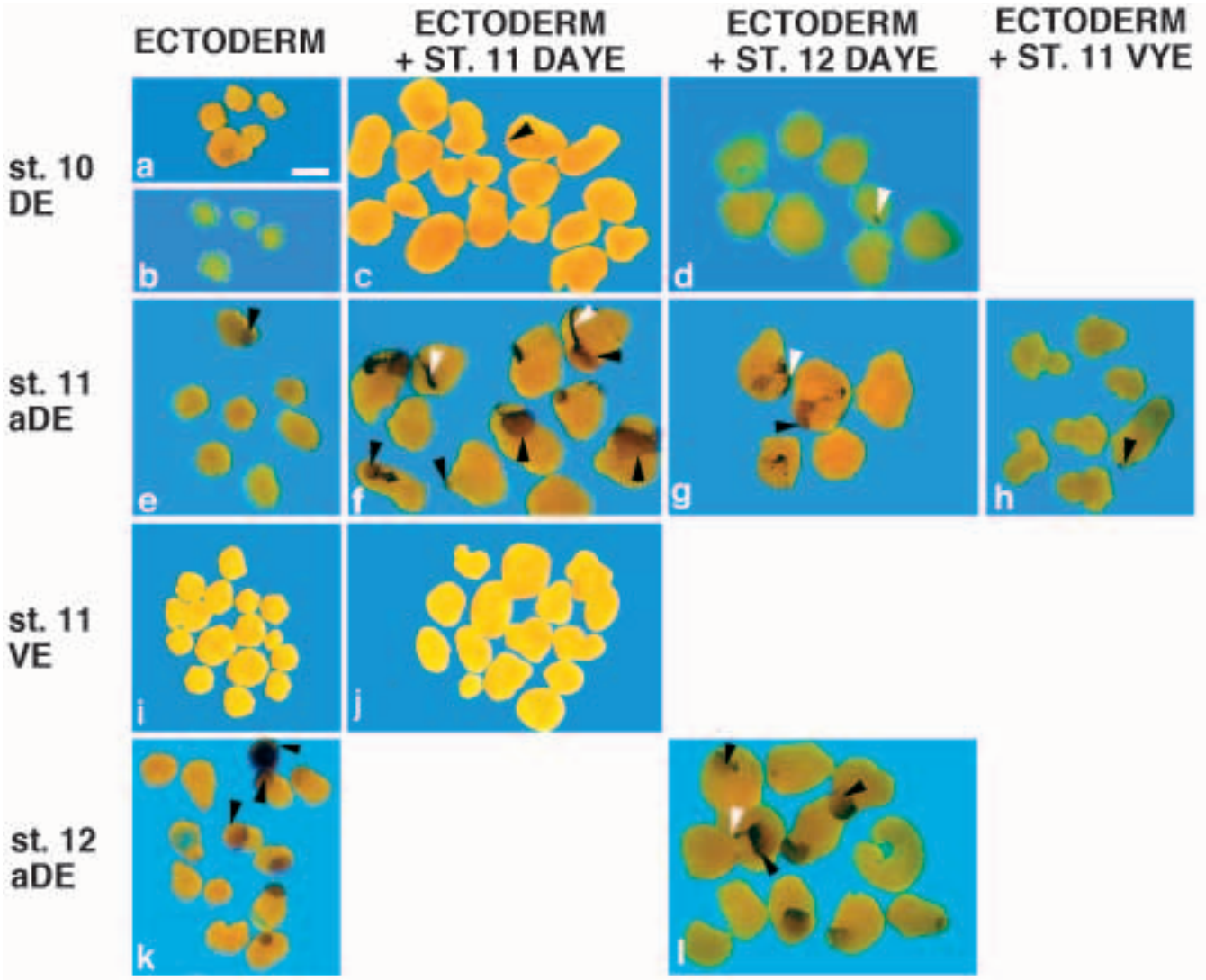


Fig. 6. Double staining in situ hybridization. Explants and stage 26 control embryos were probed with digoxygenin labeled *XCG* (in red) and fluorescein labeled *XA* (in dark blue) antisense RNA probes. (a,b) Stage 10 dorsal animal caps; (c) stage 10 animal cap conjugated to stage 11 DAYE; (d) stage 10 animal cap conjugated to stage 12 DAYE; (e) stage 11 aDE; (f) stage 11 aDE conjugated to stage 11 DAYE; (g) stage 11 aDE conjugated to stage 12 DAYE; (h) stage 11 aDE conjugated to stage 11 VYE; (i) stage 11 VE; (j) stage 11 VE conjugated to stage 11 DAYE; (k) stage 12 aDE; (l) stage 12 aDE conjugated to stage 12 DAYE. Control embryo is shown for comparison in Fig. 4 at same scale as explants. Scale bar, 200 μm, as for Fig. 4.

XA expression pattern with that of the epidermal marker *XK81* (Fig. 4b) confirmed that the *XA* transcripts were in the posterior cells of the cement gland proper and not in the epidermis that surrounds and partly engulfs the cement gland (reviewed in Sive and Bradley, 1996).

The DAYE was defined as a narrow region at the anterior limit of involution, comprising large, loosely packed cells. This piece of tissue did not express *goosecoid* RNA (not shown). The DAYE was morphologically distinct from the more posterior, smaller and more rigid prechordal plate cells that are *goosecoid* positive (Cho et al., 1991).

Initially we asked whether dorsoanterior yolky endoderm (DAYE) isolated from stage 11 or stage 12 embryos could induce cement gland in stage 10 animal caps (‘naive’ ectoderm) (Fig. 5). Explants were allowed to age until control embryos reached stage 26, and then assayed for expression of *XCG* and *XA* by double label whole-mount in situ hybridization. The results of representative experiments are shown in Fig. 6 and summarized in Fig. 7. In order to maximize the responsiveness of stage 10 ectoderm, we used dorsal animal caps that are more responsive to neural inducers than are ventral caps (Otte and Moon, 1992; Sharpe et al., 1987; Sokol and Melton, 1991). Animal caps cultured alone (Fig. 6a,b) did not express *XCG* or *XA* while those conjugated with DAYE expressed only barely visible patches of *XCG* or *XA*: in 6% of conjugates with stage 11 DAYE (Fig. 6c) and 12% of conjugates with stage 12 DAYE (Fig. 6d).

We next asked whether stage 11 (mid-gastrula) anterior dorsal ectoderm, which was partially underlain by involuting mesendoderm, was responsive to the DAYE (Fig. 5). This ectodermal substratum was weakly specified as cement gland and in the experiment shown 14% of explants formed small,

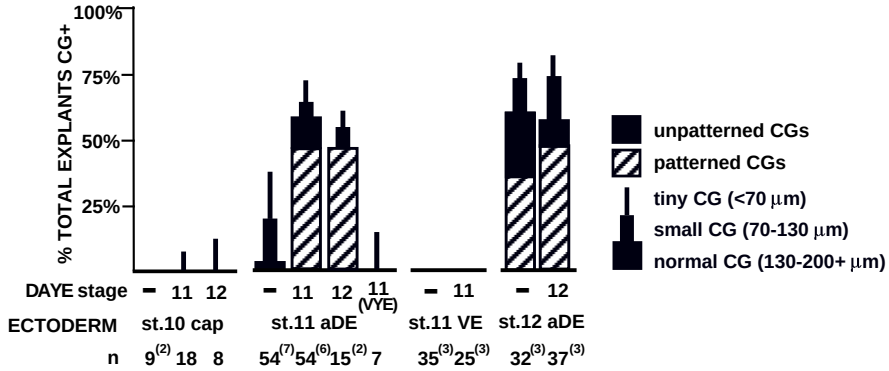


Fig. 7. Collated data from analyzing the percentage of cement gland-positive explants relative to total number of explants analyzed, the size of the explants relative to the cement gland in the whole embryo control and the percentage of explants that have patterned cement glands relative to the total number of explants. The total number of explants examined with the number of experiments performed in parentheses is given under each column.

unpatterned cement glands (Fig. 5e). We scored a cement gland as 'unpatterned' if *XA* RNA was expressed around the entire circumference of the cement gland (which expressed *XCG* RNA throughout). However, after conjugation with the DAYE that partly underlay the posterior of this tissue, 55% of explants formed cement glands (Fig. 5f; black arrows), 66% of which were very large and patterned. We scored a cement gland as 'patterned' if *XA* RNA was asymmetrically distributed such that it was expressed only within one side of the domain of *XCG* expression. Hatching gland, as scored by the characteristic star shaped *XA* expressing cells, formed in 72% of the explants (white arrows). Stage 12 DAYE also induced patterned cement glands in stage 11 anterior dorsal ectoderm (Fig. 6g), with 60% of conjugates forming cement glands, of which 66% were patterned.

The inductive capacity of the endoderm was dorsal-specific. Ventral endoderm from stage 11 embryos did not induce cement glands in stage 11 anterior dorsal ectoderm above levels seen in the control aDE (Fig. 6h). Further, neither stage 11 ventral ectoderm cultured alone (Fig. 6i), nor conjugated with stage 11 DAYE (Fig. 6j) gave rise to cement gland, showing that the enhanced response to the DAYE by stage 11 ectoderm was specific to the dorsal ectoderm.

We also tested whether stage 12 (late gastrula) DAYE was able to increase the size of cement glands formed by stage 12 anterior dorsal ectoderm (aDE). More than half of aDE explants alone from this stage formed cement glands (Fig. 6k; 54% in the experiment shown) and 42% of these were patterned. However, after co-culture with the underlying endoderm, stage 12 aDE formed cement glands that were more similar in size and shape to the cement gland in the intact embryo than those that developed from aDE alone (Fig. 6l; 75% of explants were cement gland positive, 66% of which were patterned).

The averaged results of several experiments are summarized in Fig. 7. Overall, in stage 11 anterior dorsal ectoderm cultured alone, 37% of explants formed very small, unpatterned cement glands. After conjugation to stage 11 DAYE, 74% of explants formed much larger cement glands (of which 62% were patterned) while, after conjugation to stage 12 DAYE, 60% of explants formed cement glands (of which 80% were patterned). The effect of the DAYE on stage 12 anterior dorsal ectoderm was much less pronounced with little change in the already high proportion (approximately 80%) of explants that were cement gland positive with or without DAYE.

Since the neural plate can induce cement gland (Drysdale and Ellinson, 1993), it was possible that the DAYE induced neural tissue in the stage 11 anterior dorsal ectoderm (aDE) and that this induced cement gland as a secondary effect. In order to test this hypothesis, we cultured stage 11 aDE with or

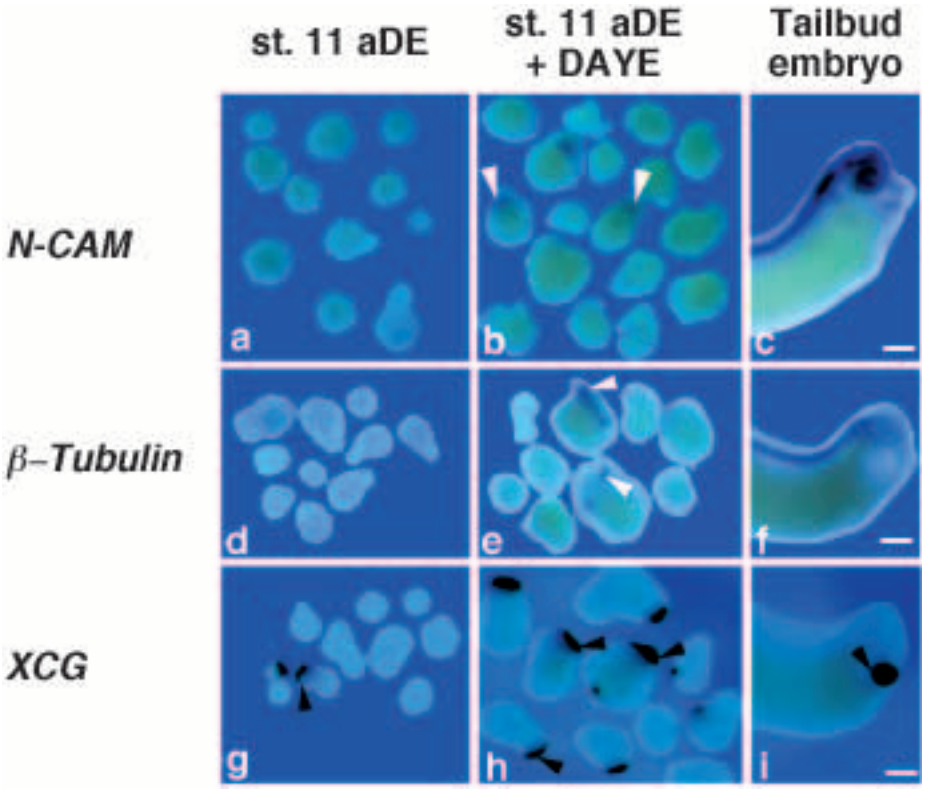
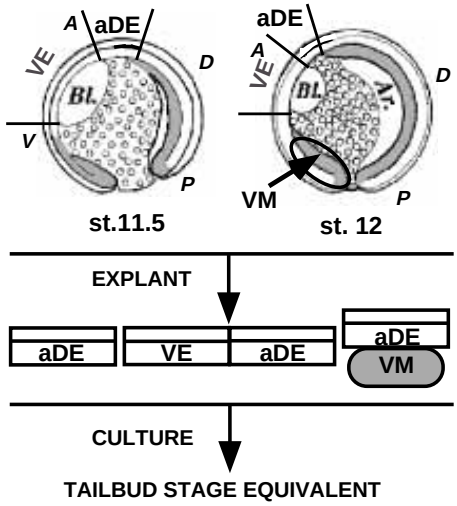


Fig. 8. In situ hybridization with neural markers. Stage 11 aDE explants alone (a,d,g), stage 11 aDE + DAYE conjugates (b,e,h) and stage 26 controls (c,f,i) were probed with digoxygenin-UTP labeled *N-CAM* (a-c), *β-Tubulin* (d-f) or *XCG* (control, g-i) antisense RNA probes. Scale bar, 200 μm.

Figs 9-11. Inhibition of cement gland formation by late gastrula ventral ectoderm and ventral mesendoderm.

Fig. 9. Dissection and explant culture scheme. Gastrula stage anterior dorsal ectoderm (aDE) was isolated and cultured alone or in conjunction with the adjacent ventral ectoderm (VE). aDE was also conjugated with ventral mesoderm (VM). All explants and conjugates were cultured until tailbud stage equivalent. See Fig. 1 for key to tissues and abbreviations.



without its cognate DAYE and tested for the expression of the neural markers *N-CAM* (Kintner and Melton, 1987) and neural-specific *β-Tubulin* (Oschwald et al., 1991). In the experiment shown, 0% of aDE explants cultured alone expressed *N-CAM* (Fig. 7a), while 29% of conjugates between the aDE and DAYE expressed low levels of *N-CAM* (Fig. 7b). Similarly, 0% of aDE explants expressed *β-Tubulin* (Fig. 7d) while 22% of conjugates between the aDE and DAYE expressed *β-Tubulin* (Fig. 7e). In the same experiment, we observed a much greater percentage of explants expressing *XCG* after conjugation to the DAYE – from 22% of aDE explants cultured alone (Fig. 7g) to 80% of aDE/DAYE conjugates (Fig. 7h). After averaging results between three experiments, in the absence of the DAYE, very few (4%, *n*=27) aDE explants expressed neural marker genes while, after conjugation with the DAYE, we observed an increase in the percentage of explants (to 28% overall, *n*=36) that expressed detectable levels of neural markers. The formation of hatching gland in a greater proportion of explants than expressed neural markers suggests that hatching gland and neural induction can be uncoupled.

These data showed that the mid-gastrula dorsoanterior yolky endoderm was a potent inducer of cement gland formation and patterning, and that this tissue required a partially induced ectodermal substratum. The data also suggested that the DAYE did not induce cement

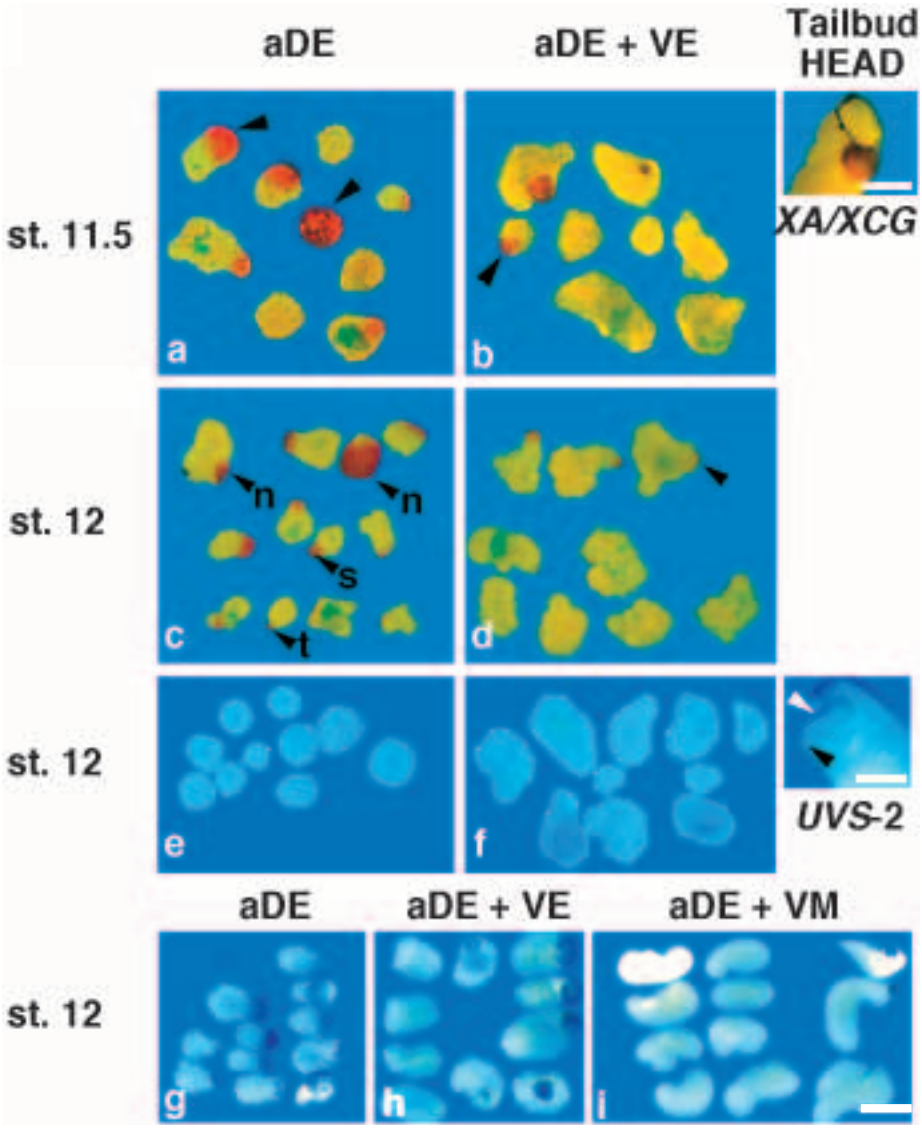


Fig. 10. In situ hybridization of explants and embryos with antisense digoxygenin-labeled *XCG* or *UVS-2* antisense RNA probes. (a-d) Explants analyzed for *XCG* (red) RNA. (a) stage 11.5 aDE; (b) stage 11.5 aDE with attached ventral ectoderm; (c) stage 12 aDE; (d) stage 12 aDE with attached ventral ectoderm. (e-f) Explants stained for *UVS-2* (blue); (e) stage 12 aDE; (f) stage 12 aDE with attached VE. (g-i) Explants stained for *XCG* (blue); (g) stage 12 aDE; (h) stage 12 aDE with attached VE; (i) stage 12 aDE conjugated with VM. Control embryos, photographed at the same magnification as explants, are shown in the right column. Black arrowheads indicate cement glands; white arrowheads indicate hatching gland; black arrows indicate cement gland sizes, defined as normal, n (130-200 μm); small, s (70-130 μm); tiny, t (<70 μm). Scale bar, 200 μm.

gland as a secondary consequence of neural induction in the aDE, but that induction was more direct.

Ventral inhibitory signals attenuate cement gland formation

Since the cement gland lies adjacent to the anterior ventral ectoderm, we asked whether the ventral ectoderm had any effect on cement gland formation. Anterior dorsal ectoderm, including specified cement gland, was isolated from stage 11.5 (late mid-gastrula) or stage 12 (late gastrula) embryos and cultured alone or with the adjacent ventral ectoderm attached (Fig. 9). Ventral ectoderm extended from the cement gland anlage to the ventral limit of the blastocoel. Explants were cultured until control embryos reached stage 26 and scored for *XCG* expression by in situ hybridization.

Data from two representative experiments are shown in Fig. 10. In the explants shown, anterior dorsal ectoderm (aDE) isolated from stage 11.5 embryos (mid-gastrula) went on to form a cement gland and express *XCG* in 88% of explants (Fig. 10a). When explants consisting of the aDE and the adjacent ventral ectoderm were cultured, 50% expressed *XCG* (Fig. 10b). Similar results were obtained after analysis of stage 12 anterior dorsal ectodermal explants, which went on to express *XCG* in 100% of explants (Fig. 10c)**;** however, in the presence of ventral ectoderm, only 56% went on to express *XCG*. The size of cement glands decreased markedly after culture with attached ventral ectoderm. In the experiment shown (corresponding to experiment B in Fig. 11), the average size of cement glands formed from the stage 12 explants (Fig. 10c,d) was 96±59 μm without ventral ectoderm, and 58±25 μm in the presence of ventral ectoderm. Stage 12 explants were not specified as hatching gland, as shown by absence of *UVS-2* expression (Fig. 10e), nor was hatching gland induced in the explants by ventral ectoderm (Fig. 10f).

The results of 16 experiments are summarized in Fig. 11. Within each experiment, in the presence of the ventral ectoderm, both the number of cement-gland-positive explants and the size of the explants consistently declined relative to controls cultured without ventral ectoderm. On average, the number of stage 12 aDE explants that went on to express *XCG* decreased by 31% in the presence of ventral ectoderm. The size of cement glands in the presence of ventral ectoderm also consistently decreased. Cement gland size was measured, along the longest diameter, and classified as tiny (<70 μm), small (70-130 μm) or normal (130-200 μm; the cement gland in whole embryos is 180-200 μm wide). In the presence of ventral ectoderm, the proportion of cement gland-positive explants that formed tiny cement glands increased 250% relative to the proportion of cement gland-positive explants that formed tiny cement glands without attached ventral ectoderm.

We also asked whether the ventral mesendoderm was able to inhibit cement gland formation. Conjugates between stage 12 anterior dorsal ectoderm and stage 12 ventral mesendoderm were cultured until controls reached stage 26, before being assayed for *XCG* expression (Fig. 9). In order to compare the relative strength of cement gland inhibition by the ventral ectoderm and ventral mesendoderm in the same experiment, aDE explants were also made with the ventral ectoderm attached. In the experiment shown in Fig. 10g-i, and summarized in Fig. 11e; 100% of explants from stage 12 embryos went on to express *XCG* (Fig. 10g). 66% of explants including ventral ectoderm went on to express *XCG* (Fig. 10h), but only 28% went on to express *XCG* after conjugation to the ventral mesendoderm (Fig. 10i). Those explants that did express *XCG*

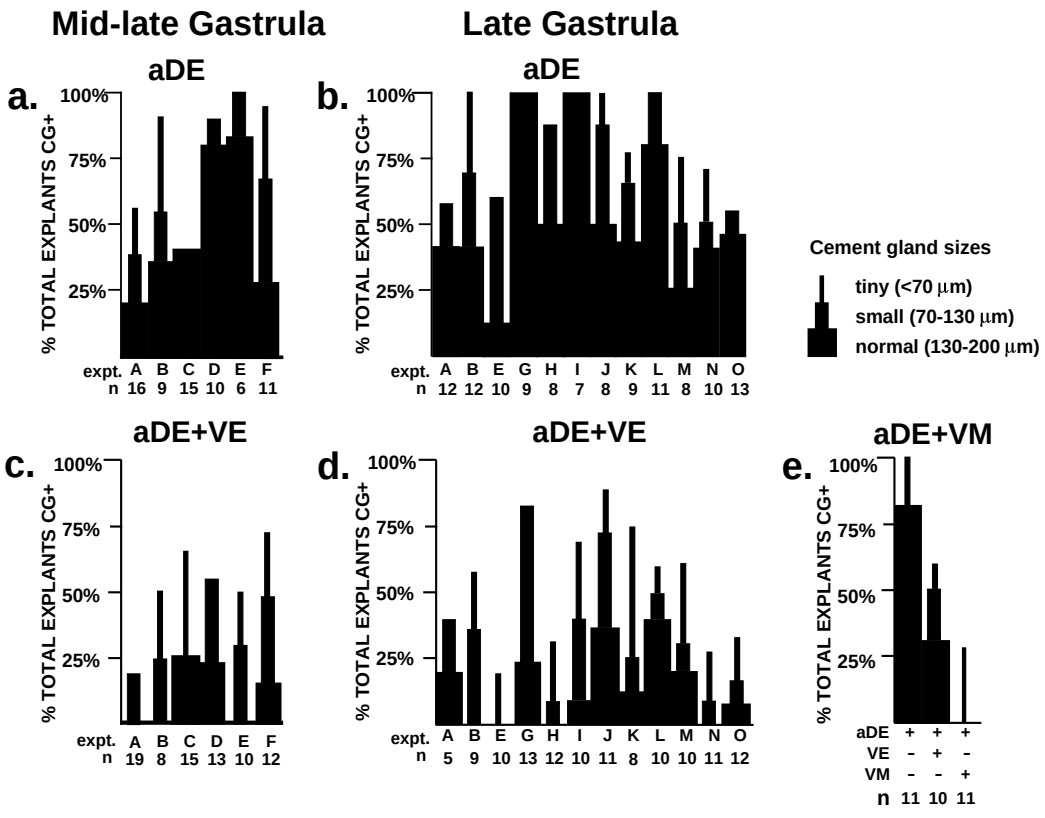


Fig. 11. Collated data is shown from sixteen experiments analyzing anterior dorsal ectoderm (aDE) with or without attached ventral ectoderm (VE) and one experiment analyzing aDE with or without conjugation to ventral mesendoderm (VM). Two additional experiments of aDE with or without VM have been performed with very similar results, although these were not performed in comparison with aDE plus ventral ectoderm. The percentage of *XCG*-positive explants relative to the total number analyzed and the size of the explants relative to the cement gland in the whole embryo control are shown. The sample size (n) for each of experiment (labeled A through O) is given under each column. Explants were analyzed for *XCG* and *XA* RNAs together (A-D), or *XCG* RNA alone (E-O). *XA* localization within the reduced cement glands was not easy to observe and was not scored.

after conjugation to ventral mesendoderm formed only tiny (<70 μm diameter) cement glands.

In summary, these data showed that ventral tissues can act late during gastrulation, after the cement gland primordium has been induced, to attenuate cement gland differentiation. The ventral ectoderm incompletely but consistently inhibited cement gland formation, while the ventral mesendoderm was a very potent and often complete inhibitor of cement gland differentiation.

DISCUSSION

We have addressed the timing of cement gland induction and have documented previously undescribed cell interactions that modulate *Xenopus* cement gland formation. Firstly, we have shown that the outer layer of the embryonic ectoderm, from which the cement gland forms, becomes specified as cement gland only by mid-gastrula. Prior to this, the inner ectodermal layer, which does not contribute to the mature cement gland, is strongly specified as cement gland. Secondly, we have demonstrated for the first time that the dorsoanterior yolky endoderm (DAYE) is a potent cement gland inducer that can act on partially induced dorsal ectoderm. Thirdly, we have shown that both the ventral ectoderm lying adjacent to the cement gland primordium and the more distant ventral mesendoderm attenuate cement gland formation. This is the first demonstration that normal ventral tisues can inhibit cement gland differentiation. The DAYE and ventral ectoderm directly abut the cement gland primordium and are likely to be modulators of cement gland formation in the whole embryo. These data highlight the complexity of inducing even a simple organ such as the cement gland.

Induction of the true cement gland primordium

By analyzing cement gland induction specifically in the outer ectodermal layer, we gained a clearer picture of the timing of cement gland induction than has been obtained previously. We consider that mid-gastrula, the time that the outer ectoderm becomes specified as cement gland, is the first time that the true cement gland primordium is induced. At early gastrula, when explants comprising both layers of the posterior dorsal ectoderm (presumptive neurectoderm) form a cement gland (Sive et al., 1989; Fig. 2), we think that cement gland arises as a result of secondary inductive interactions between the inner and outer layers.

Why is the outer layer not specified as cement gland before mid-gastrula? We envisage three possibilities. Firstly, cement gland inducing signals may not be present in the embryo until that time. It is not clear when endoderm, mesoderm or neurectoderm first aquire their inducing capacities; however, by mid-gastrula the dorsoanterior endoderm is clearly an active cement gland inducer. Neurectoderm is also specified by this time (Saha and Grainger, 1992; Sharpe, 1991; Sive et al., 1989) and may be capable of inducing cement gland (Drysdale and Elinson, 1992), although this has not been tested. A second possibility is that the outer ectodermal layer is not competent to respond to inducers until mid-gastrula. In support of this, we showed that the intact dorsal ectoderm could not be induced to form cement gland by the dorsoanterior endoderm until mid-gastrula. Thirdly, inducing signals may be present in the embryo earlier, but may not reach the outer layer until mid-gastrula. Previous data have shown that the inner ectoderm attenuates the strength of cement-gland-inducing signals that reach the outer layer from the dorsal mesoderm (Drysdale and Ellinson, 1993). This layer may therefore also increase the time it takes for sufficient inducer to reach the outer layer and induce cement gland.

Interactions between the inner and outer ectodermal layers

The observation that early gastrula inner ectoderm is specified as cement gland agrees with morphological data from Asashima and Grunz (1983). A major question arising from this result is why the intact animal cap does not go on to express cement gland markers. While we cannot entirely rule out effects of culture conditions, we propose that, in the early gastrula, an inhibitory signal from the outer ectodermal layer may prevent cement gland differentiation from occurring in the inner layer, where it would not be useful.

The cement gland that differentiates from the inner layer is ‘atypical’ with multiple foci of strong cement gland marker gene expression and abnormal cell morphology, where cement gland cells are sticky but apparently not as elongate as outer layer-derived cement gland cells (Asashima and Grunz, 1983). Further, in the intact embryo, cement gland arises only from the dorsal ectoderm whereas, in early gastrulae, both dorsal and ventral inner layers are specified as cement gland. This latter point suggests that cement gland that differentiates from the inner layer may not be induced by dorsally derived signals, but may reflect a more general ectodermal ‘priming’ process, that can be realized as cement gland differentiation in explants.

In mid-gastrula stage explants comprising both ectodermal layers, posterior dorsal mesoderm (presumptive notochord) is able to suppress cement gland formation, with concomitant activation of neural markers (Sive et al., 1989, 1990). This suggests that, in the whole embryo, the inner layer loses cement gland specification as it becomes reprogrammed by the dorsal mesoderm to a neural fate at mid-gastrula. Concordantly, the outer layer acquires cement gland specification. One possibility is that this occurs because the inner layer has acquired cement-gland-inducing capacity. In support of this, previous studies have shown that the early neurula inner ectodermal layer, that is, the anterior neural plate, can induce cement gland in early gastrula outer ectoderm (Drysdale and Elinson, 1992), however this activity has not been tested during gastrulation.

Synergy between the dorsoanterior yolky endoderm and other inducers

The dorsoanterior yolky endoderm is a potent cement gland inducer, but requires a partially induced ectodermal substratum on which to act. This implies that at least two inducing tissues synergistically activate cement gland formation in the embryo. The responsive mid-gastrula aDE was not specified as neurectoderm, but had clearly undergone partial induction, presumably by the dorsal mesoderm. This may reflect an early inductive step in the dorsal ectoderm that is part of both cement gland and neural specification. Cement gland can be induced by dorsoanterior endoderm without concomitant neural differentiation, showing that overt neural induction is not obligatory for cement gland induction. Since early gastrula

dorsal ectoderm did not respond to the endoderm (Fig. 6 and Sive et al., 1989), the competence to respond to the DAYE appears to be distinct from the greater competence for neural induction of early gastrula dorsal ectoderm relative to ventral ectoderm observed in other assays (Otte and Moon, 1992; Sharpe, 1987; Sokol and Melton, 1991). By late gastrula, the DAYE may have largely completed its inductive role, since specified cement gland ectoderm (aDE) isolated from late gastrula embryos was much less responsive to the DAYE than was mid-gastrula aDE.

Our data parallel those of Dixon and Kintner (1989) who showed that the anterior mesendoderm (including the prechordal plate) was able to induce neural markers only in combination with planar signals from a Keller sandwich. Yamada (1938) showed that, in *Rana nigromaculata*, a piece of tissue including both the prechordal plate and dorsoanterior yolky endoderm could induce cement gland in an Einsteck assay. Since Yamada did not test the DAYE alone, the results presented here are not directly comparable to his.

What factors might the dorsoanterior endoderm be secreting that could induce cement gland? Cephalic hedgehog (*chh*) mRNA is present in neurula stage endoderm and, when expressed at high levels in animal caps, is able to induce cement gland, but not neural markers tested (Ekker et al., 1995; Lai et al., 1995). The distribution of *chh* at mid-gastrula is not known, and it is probable that the endoderm expresses other, unidentified cement-gland-inducing factors. Although *chordin*, *noggin* and *follistatin* are not expressed more anteriorly than the prechordal plate, it is possible that their protein products diffuse forward to effect cement gland induction. These factors induce cement gland in early gastrula animal caps, although all also induce neural tissue and may therefore induce cement gland indirectly (Hemmati-Brivanlou et al., 1994; Lamb et al., 1993; Sasai et al., 1994).

Ventral inhibitors attenuate cement gland induction

Since the ventral ectoderm lies directly adjacent to the cement gland primordium, we conclude that inhibitory signals from this tissue normally compete with dorsally derived inducing signals to limit the size of the cement gland and the precise position at which it forms. The ventral inhibitory signals that we defined can clearly act after cement gland has been induced in the outer ectoderm, revealing a late event in cement gland determination. Whether similar signals act earlier during gastrulation as the cement gland primordium becomes induced in the outer ectodermal layer is currently unclear.

We observed attenuation of cement gland formation by ventral tissues only when these were attached to small dorsal ectodermal explants. When larger dorsal ectodermal explants, which included presumptive neural plate, were assayed, little inhibition of cement gland differentiation by the ventral ectoderm was observed (not shown), suggesting that the inhibitory effects were overcome by dorsally derived signals. The ventral mesendoderm, which was a more potent inhibitor of cement gland than the ventral ectoderm, never abuts the cement gland primordium. However, signals from this region could diffuse dorsally to play an active role in preventing cement gland differentiation in ventral regions. It is unclear whether ventral signals actively inhibit cement gland inducers, or whether ventral tissues comprise a passive sink for dorsally derived inducers. As far as the embryo is concerned, either mechanism would be an effective way of dampening dorsal signals.

What does the ventrally inhibited cement gland ectoderm become? One possibility is that more posterior dorsal ectodermal tissues form. We could rule this out since the hatching gland marker *UVS-2* (Sato and Sargent, 1990) was not expressed after cement gland inhibition. A second possibility is that inhibited cement gland becomes epidermis. *XK81*, an epidermal cytokeratin marker (Sargent et al., 1986) is excluded from the cement gland (Fig. 4) and we asked whether the area of the explant not expressing *XK81* decreased after ventral inhibition. However, this determination could not be made accurately enough to reach a firm conclusion (data not shown). Thirdly, the presumptive cement gland cells may die in response to ventral inhibitory signals and we have not yet addressed this possibility.

The ventral mesendoderm prevents expression of the homeodomain gene *XANF-1* from the inner layer of mid-gastrula anterior dorsal ectoderm (Zaraisky et al., 1992) possibly reflecting the same inhibitory activity that we observe on cement gland formation. Kato and Gurdon (1994) observed a strong inhibition of muscle differentiation by ventral ectoderm. Although the inhibition of muscle formation by ventral ectoderm occurs at a time similar to the effect on cement gland that we observe, the ventral mesendoderm does not attenuate muscle formation, suggesting that the mechanism by which ventral tissues inhibit ectodermal (cement gland) and mesodermal (muscle) fates is different, or that different activities in the ventral ectoderm and mesendoderm can both suppress cement gland differentiation.

Two classes of factor that are expressed ventrally and are candidates for suppressing cement gland differentiation are wnt proteins and Bone Morphogenetic Proteins (*BMPs*). *Xwnt-8* is expressed from early gastrula in the ventrolateral mesendoderm (Christian and Moon, 1993) and is a candidate for the mesendoderm-derived activity that may be able to act directly on the ectoderm (Lustig and Kirschner, 1995). Misexpression and loss-of-function studies have shown that *BMP-4* and *BMP-7* can act on early gastrula stage ectoderm to prevent cement gland formation (Hawley et al., 1995; Sasai et al., 1995). During later gastrula stages, when we observed cement gland inhibition by ventral tissues, these *BMPs* are expressed in the ventral ectoderm, suggesting that they could also act at this time to inhibit cement gland formation.

Timing cement gland induction: sequential and competing signals

Together, these data suggest that induction of the cement gland in the outer ectodermal layer involves sequential and competing signals that are summarized in Fig. 12. Early during gastrulation, a general ‘priming’ event in the inner layer of the animal cap takes place (this work). Although the purpose of this priming may be to sensitize the ectoderm to subsequent induction, this event can be manifest as cement gland differentiation in a specification assay (this work; Asashima and Grunz, 1983). This cement gland potential of the inner layer is not realized because the overlying outer ectoderm inhibits cement gland differentiation in the inner layer.

By mid-gastrula, cement gland has been induced in the outer ectodermal layer, from which the cement gland will eventually differentiate (this work). The inducing signals are likely to

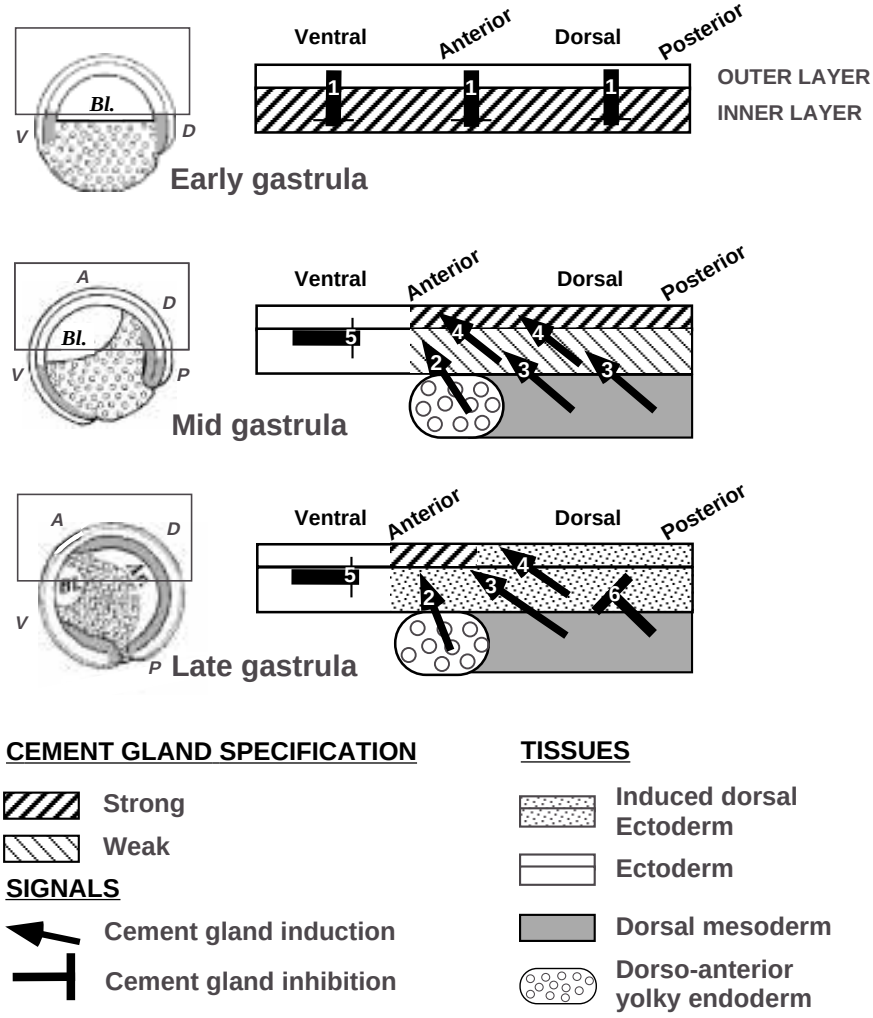


Fig. 12. Model for *Xenopus* cement gland induction. Staged embryo diagrams are shown with boxed areas schematically enlarged to the right of each embryo. For key to tissues and abbreviations see Fig. 1. Early during gastrulation (stage 10), the inner ectodermal layer of the ectoderm is specified as 'atypical' cement gland. This layer is prevented from realizing its fate and differentiating as cement gland by an inhibitory signal from the outer ectoderm (1). By mid-gastrula (stage 11-11.5) the inner ectoderm remains weakly specified as cement gland dorsally as it becomes induced towards a neural fate. In addition, the anterior dorsal ectoderm has become responsive to inducers from the anterodorsal yolky endoderm (2). These signals may synergize with inducing signals from the anterior dorsal mesoderm (3) and from the adjacent neurectoderm (4) to induce cement gland in the outer ectodermal layer. Cement gland specification is not yet restricted to the anterodorsal outer ectoderm (data not shown). Ventrally derived inhibitory signals (5) acting late during gastrulation position the front of the cement gland and may prevent cement gland from differentiating ventrally. Signals from the posterior (chordal) mesoderm inhibit cement gland formation posteriorly (6). By late gastrula, cement gland is beginning to differentiate anteriorly.

arise from the dorsoanterior yolky endoderm and the inner layer of the dorsal ectoderm after it has been induced to a neural fate (this work; Drysdale and Elinson, 1993; Itoh and Kubota, 1991; Yamada, 1938; reviewed in Sive and Bradley 1996). However, our data suggest that neural specification is not required for cement gland induction. In addition, more distal, mesodermally derived signals may diffuse forward to act on the cement-gland-forming region (Sive et al., 1989). Inducers from both the dorsal mesoderm and the dorsoanterior yolky endoderm must pass through the inner ectoderm before inducing the outer layer and may be attenuated during this passage (Asashima and Grunz, 1983; Drysdale and Elinson, 1993). Cement gland induction becomes anteriorly limited by late gastrula when cement gland-specific gene expression begins, perhaps by inhibitory signals derived from the notochord (Sive et al., 1989, 1990).

From mid- to late gastrula, and possibly later, the ventral ectoderm antagonizes cement-gland-inducing signals (this work). Clearly, dorsal signals 'win' since a cement gland forms, but ventral signals may limit the size of the cement gland and delineate the position at which it forms. Cement gland induction seems complex. A full understanding of this process will require definition of the spectrum of secreted factors and genes that are involved.

This study was started many years ago, and we acknowledge the contribution of Brenda Kennedy in analyses of inner/outer ectodermal specification and for initial attempts to examine the role of the ventral ectoderm during cement gland induction. We thank members of our laboratory for thoughtful comments and Vlademir Apekin for expert frog care. We thank Igor Dawid, Chris Kintner, Tom Sargent and Sheryl Sato for plasmids. This work was supported by a grant from the NSF (95-13976) and by a NSF Young Investigator Award to H. S., who was the recipient of a Searle Scholar Award from the Searle Scholars Program /Chicago Community Trust and is the Latham Family Career Development Professor at MIT. L. B. was supported by a SERC/NATO postdoctoral fellowship and D. W. by a Howard Hughes Medical Institute predoctoral fellowship.

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