Development 129, 4387-4397 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV5019

Cement gland-specific activation of the *Xag1* promoter is regulated by co-operation of putative Ets and ATF/CREB transcription factors

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Accepted 21 June 2002

SUMMARY

The cement gland marks the extreme anterior ectoderm of the *Xenopus* embryo, and is determined through the overlap of several positional domains. In order to understand how these positional cues activate cement gland differentiation, the promoter of *Xag1*, a marker of cement gland differentiation, was analyzed. Previous studies have shown that *Xag1* expression can be activated by the anterior-specific transcription factor Otx2, but that this activation is indirect. 102 bp of upstream genomic *Xag1* sequence restricts reporter gene expression specifically to the cement gland. Within this region, putative binding sites for Ets and ATF/CREB transcription factors are both necessary and sufficient to drive cement gland-specific expression, and cooperate to do so. Furthermore, while the putative ATF/CREB factor is activated by Otx2, a factor acting through the putative Ets-binding site is not. These results suggest that Ets-like and ATF/CREB-like family members play a role in regulating *Xag1* expression in the cement gland, through integration of Otx2 dependent and independent pathways.

Key words: Xag1, *Xenopus*, Anterior, Ectoderm, Transgenic, Promoter analysis, Ets, ATF/CREB, Cement gland

INTRODUCTION

The cement gland of the frog, *Xenopus*, is a simple mucus-secreting epithelium that anchors the newly hatched tadpole to a solid surface. It begins to differentiate at the end of gastrulation, from ectoderm that lies at the extreme anterior of the embryo, in front of the neural plate. In *Xenopus*, the outer layer of ectoderm in this region forms the cement gland primordium, while the inner layer forms part of the stomodeal primordium. The cement gland defines a conserved position in all vertebrate embryos, at the anterior where embryonic ectoderm and endoderm touch. Its position and early differentiation make the cement gland a useful marker for analyzing anterior positional information, and afford the unusual opportunity of tracing an organ from its determination to its differentiation (Sive and Bradley, 1996).

Several lines of evidence lead us to propose that the cement gland is positioned through the combination of several instructions (reviewed by Wardle and Sive, 2002). A key step in cement gland formation involves overlap of a domain that defines anterodorsal position (AD) with a domain that defines ventrolateral position (VL) (Gammill and Sive, 1997). As the cement gland forms only in the outer layer of ectoderm and the AD and VL domains span more than one germ layer, they must be superimposed on a domain that defines outer layer ectodermal fate (EO). Cement gland fate is therefore a summation of AD, VL and EO domains (AD + VL + EO=CG; see Fig. 7).

The AD domain appears to be defined by Otx2, a paired class homeobox protein expressed in anterior ectoderm and mesendoderm. Otx2 is sufficient to activate cement gland and anterior neural gene expression when ectopically expressed (Gammill and Sive, 1997; Blitz and Cho, 1995; Pannese et al., 1995; Gammill and Sive, 2001). Otx2 is also necessary for anterior determination, as a dominant negative version of Otx2 (Otx2-Engrailed) prevents formation of the cement gland and other head structures in *Xenopus* (Gammill and Sive, 2001; Isaacs et al., 1999), consistent with knockout data in mice (Ang et al., 1996; Acampora et al., 1995; Matsuo et al., 1995).

Otx2 can activate cement gland fates only in ventrolateral ectoderm and not in the neural plate, thereby defining a ventrolateral (VL) domain permissive for cement gland formation (Gammill and Sive, 1997). This VL domain expresses high levels of BMP4 in ectoderm and mesendoderm, and may be defined by this protein or some downstream consequence of BMP signaling (Gammill and Sive, 2000).

As both *otx2* and *bmp4* are expressed in more than just the ectodermal germ layer, an additional factor(s) must restrict the cement gland determination activity of these genes to the ectoderm, and specifically to the outer ectodermal layer. Selection of outer ectodermal layer fate (O) has occurred by mid-gastrula in a process that may involve suppression of cement gland fate in the inner layer (Bradley et al., 1996). However, the factors involved in defining ectodermal identity and outer ectodermal layer specificity are unknown (Chalmers et al., 2002).

Factors that define each of the AD, VL and EO domains must directly or indirectly interact to determine the cement gland primordium, and to activate differentiation genes such as *Xcg1*, *Xag1* and *Xa1* (Sive et al., 1989; Sive and Bradley, 1996). In order to begin to ask how domain-specific factors work together to direct cement gland differentiation, we have analyzed the *Xag1* promoter. *Xag1* encodes a protein that is likely to be retained in the endoplasmic reticulum, which may aid protein secretion and is the pioneer gene in the Agr family (D. H. W. and H.L.S., unpublished). It is expressed in both the hatching gland and cement gland. We show that elements in the *Xag1* promoter that may bind members of the Ets and ATF/CREB transcription factor families are both necessary and sufficient to direct reporter gene expression specifically to the cement gland, but not hatching gland. In addition our results confirm that both Otx2-dependent and Otx2-independent pathways are involved in activation of *Xag1* expression.

MATERIALS AND METHODS

Transgenic *X. laevis* embryos

Transgenic embryos were generated as described (Kroll and Amaya, 1996) with the following modifications. Protease inhibitors were omitted during egg extract and sperm nuclei preparation. Digitonin (Roche) dissolved in DMSO was substituted for lyseolecithin during sperm nuclei preparation. Sperm nuclei preps were slow frozen at –20°C overnight then transferred to –80°C. During the final step of egg extract preparation, the extract was heated to 80°C for 10 minutes, microcentrifuged and the cleared supernatant frozen in liquid nitrogen. For each reaction 2×10^5 sperm nuclei were incubated with 1 μg linearized DNA in a total of 5 μl for 5 minutes, then added to a mix of 10 μl SDB and 2.5 μl egg extract. Nuclei were then diluted 1:50 in MOH (Offield et al., 2000) and injected using a Harvard 11 infusion pump. In later experiments, egg extract was omitted with no decrease in the frequency of transgenic embryos.

Scoring Transgenic embryos for cement gland-specific expression

Embryos expressing *gfp* in the cement gland were scored as positive, those embryos that did not show expression in the cement gland were scored as negative. These scored embryos did not show transgene expression outside the cement gland. Some transgenic embryos showed small patches of strong, superficial staining for *gfp*. This staining is reminiscent of expression seen when plasmid DNA is injected and can be seen with all constructs, including control constructs (this study) and those for other promoters, such as mfy5 (Polli and Amaya, 2002). Such staining is easily distinguishable from normal transgene expression and, as such, embryos with this type of expression were included in scoring for cement gland expression of *gfp*.

Scores for each construct were tabulated and, for Figs 3 and 4, assigned to groups according to the following scheme: +++, cement gland expression of *gfp* in more than 25% of embryos; ++, 18-25%; +, 9-17%; +/–, 2-8%; –, less than 2%. These ranges were chosen to represent our experience that strong promoters drive expression in 25% or more of embryos (see also Kroll and Amaya, 1996; Sparrow et al., 2000; Davis et al., 2001). We assign a value of less than 2% to represent background expression, possibly owing to the random integration of multimerized constructs recapitulating lost sites or the integration site acting as a gene trap. In support of this, throughout the course of all these experiments (in which 4178 embryos were scored) we have on a small number of occasions (up to 15 embryos) seen expression of *gfp* in tissues such as the somites, lateral mesoderm, the eye and regions of the brain; these embryos were not included in the scoring.

Cloning *Xag1* genomic sequences

The *Xag1* genomic sequence used in this study had been previously isolated by B. Kennedy. An 8.5 kb region including 5.75 kb of upstream sequence and 2.75 kb of *Xag1* introns and exons was cloned in to *Eco*RI site of pBluescript SK–.

Rapid amplification of cDNA 5′ ends (5′ RACE)

5′ RACE was performed using GibcoBRL Life Sciences kit according to manufacturer's instructions, with two exceptions: (1) first strand cDNA synthesis primers were annealed to the template mRNA for 20 minutes at 70°C, followed by slow cooling to 55°C before being placed on ice; (2) the reaction time of TdT poly-dC tailing of the first-strand cDNA was limited to 2.5 minutes. All RACE PCRs underwent 30-35 cycles of 1 minute at 94°C, 1 minute at 60°C and 2 minutes at 72°C, with a 'hot start', as described by the manufacturer.

Primers used:

Anchor primer (I=inosine), 5′ GCTACTCGAGTAACGGG-IIGGGIIGGGIIG;

oligo dT first strand cDNA primer, 5′ TGCGACTCGAG-TTTTTTTTTTTT;

Xag1 first strand cDNA synthesis primer, 5′ TGAGCACA-GGAGGACAAG;

Xag1 first nested PCR primer, 5′ CGTTTCTAGAAGCC-TGCATTATGTCTGTGG; and

Xag1 second nested PCR primer, 5′ GCTTCTAGAATGTCC-TGATCCTTTTAGTC

This 5′ RACE analysis yielded two classes of transcripts. A single round of PCR amplification resulted in a pool of products, all of which begin at an initiator element 25 bp downstream of a TATA box. Further amplification of this PCR product pool, using primers upstream of the TATA box, yielded a new pool of cDNAs, each of a slightly different length extending 100-150 bp upstream of the TATA box. Both of these classes of transcripts were represented in the cDNA library used for the original identification of a full-length *Xag1* transcript.

Xag1 promoter constructs

8kbXag.nGFP: GFP containing a nuclear localization signal and including globin 5′ and 3′ sequences was excised from CMVnGFP (Kroll and Amaya, 1996) with *Hin*dIII and *Not*I (blunted) and cloned into the *Bse*RI site (blunted) of *Xag1* that lies 36 bp downstream of transcription start site.

–275Xag.nGFP: 8kbXag.nGFP was cut with *Eco*RV upstream of the transcription start site and with *Bst*U1 downstream of nGFP cassette and ligated into the *Sma*I and *Hinc*II sites of pBluescript SK. Further deletion constructs were generated by PCR using GFP.L (5′AAAGGGCAGATTGTGTGGAC) and an upper primer (listed below) containing a *Not*I site (underlined). PCR products were cloned into *Not*I/*Bam*HI site of –275bp.nGFP.

–161 bp.U: 5′GGTGGCGGCCGCAAGGAAAAGTATG

–102 bp.U: 5′GGTGGCGGCCGCAAGACTAAAAGGATCAGG

–73 bp.U: 5′CTGGTGCGGCCGCTGACGTTGATCTCTAGC

TATA.U: 5′GCAGTTAGCGGCCGCTTGGGTATA

Linkerscan replacements were made to cover a consecutive series of 14 bp regions upstream of the transcription start site, except linkerscan 8, which replaces a downstream putative GATA-binding site of 4 bp. Linkerscan constructs in –275bpXag.nGFP were made using the QuickChange site directed mutagenesis kit (Stratagene), according to manufacturer's directions. The QuickChange protocol uses two primers, the exact reverse and complement of each other. The primer corresponding to the coding strand is given below:

linkerscan 1, 5′ GGTTGGGTCAAATCTAGATCACTTCTAT-CGACATCCTGG;

linkerscan 2, 5′ CTAAAAGGATCTAGAACGAAGTTGATT-AAGGCTGAC;

linkerscan 3, 5′ GACATCCTGGTTAGCGAATTCTTTGG-TCTCTCTAGCAGTTA;

linkerscan 4, 5′ CTGACGTTGAGAATTCTACTGGCTACC-TGCTTTGG;

linkerscan 5, 5′ CTCTAGCAGTTAGTCTCGAGAATAGTAT-AAATACACCAC;

linkerscan 6, 5′ CTGCTTTGGCTCTTAGAATTCCACCACCTG;

lnkerscan 7, 5′ GGTATAAATACATAGTTAGAATTCGTCATCA-GCATTATCTCAG;

lnkerscan 8, 5′ GCAGCATTACTCGAGAGGAGC.

–275bpLS2.EBSmut: the QuickChange kit was used to mutate the two distal EBS. The primer corresponding to the coding strand is given (mutations underlined). Distal site, 5′ CTTGA-CACATCAAAGGCAGACTTGCAGGCAGG; proximal site, 5′ GCCTAAAGAAAAAGGCAAGTATGATATGGG.

–102bpXag.nGFP linkerscan constructs 3-8 were generated by PCR using –102bpXag and GFP.L as upper and lower primers, and –275bpXag.nGFP linkerscan constructs (3-8) as templates. *Not*I/*Bam*HI fragments were then cloned into –102bpXag.nGFP.

–102bpXag.nGFP linkerscan constructs 1-2 were made as above except the following upper primers were used (*Not*I site underlined):

–102 bp linkerscan 1, 5′GGTGGCGGCCGCTCTAGATCAC-TTCTATCG;

–102 bp linkerscan 2, 5′ GGTGGCGGCCGCAAGACT-AAAAGGATCTAG.

Multimerized cassette constructs were generated using the following oligonucleotides, which were annealed, filled in with Klenow, cut with *Not*I/*Sac*II and cloned into *Not*I/*Sac*II site of TATA.nGFP (*Not*I/*Sac*II sites underlined):

5xreg1, 5′ CTTGACCGCGGAGACTAAAAGGATCAGACT-AAAAGGATCAGACTAAAAGGATCAGACTAAAAGGATCAGAC-TAAAAGGATCGCGGCCGC;

5xEBS, 5′ CTTGACCGCGGAGGACATCCTGGTTAGGACA-TCCTGGTTAGGACATCCTGGTTAGGACATCCTGGTTAGGACA-TCCTGGTTGCGGCCGC;

5xCRE, 5′ CTTGACCGCGGTTAAGGCTGACGTTTTAAGG-CTGACGTTTTAAGGCTGACGTTTTAAGGCTGACGTTTTAAGG-CTGACGTTGCGGCCGC;

5xreg5, 5′ CTCGACCGCGGATACCTGCTTTGGGATACC-TGCTTTGGGATACCTGCTTTGGGATACCTGCTTTGGGATACC-TGCTTTGGGGCGGCCGC;

5xEBSmut, 5′ CTTGACCGCGGAGGACGCCTTGGTTAGG-ACGCCTTGGTTAGGACGCCTTGGTTAGGACGCCTTGGTTAG-GACGCCTTGGTTGCGGCCGC;

5xCREmut, 5′ CTTGACCGCGGTTAAGGCTGTGGCTTTAA-GGCTGTGGCTTTAAGGCTGTGGCTTTAAGGCTGTGGCTTTA-AGGCTGTGGCTGCGGCCGC;

3EBS/2CRE, 5′ CTTGACCGCGGAGGACATCCTGGTTAGG-ACATCCTGGTTAGGACATCCTGGTTTTAAGGCTGACGTTTTA-AGGCTGACGTTGCGGCCGC.

MLP constructs were generated by inserting the *Hin*dIII/*Bam*HI fragment (blunted) of MLP-PTCAT (L. Gammill, unpublished), which contains the Adenovirus Major Late Promoter, into the *Not*I/*Bse*R1 (blunted) site of 5xEBS, 5xCRE or 3EBS/2CRE.nGFP. This removes the *Xag1* TATA box, transcription start site and 5′UTR and replaces them with MLP. All constructs were verified by sequencing before use.

Constructs were linearized with *Not*I (deletion and linkerscan constructs), *Sac*II (multimerized cassette constructs and MLP constructs) or *Sal*I (8kbXag.nGFP), purified using GeneClean (Bio101) and diluted to give 200-250 ng/μl. MLPonly.nGFP was generated by cutting 5xEBS.MLP with *Pst*I and *Sac*II, which excises the 5xEBS cassette. The MLPonly.nGFP band was purified from a gel using GeneClean. All constructs were prepped and tested at least twice in at least three separate transgenic experiments.

In situ hybridization

Embryos were collected at stages indicated in the text and processed for in situ hybridization as described (Sive et al., 2000). AntiGFP probe was made by linearizing TATA.nGFP construct with *Bam*HI and transcribing with T7 RNA polymerase in the presence of digoxygenin-UTP (Roche) as described (Sive et al., 2000). In most cases, in situ hybridization was used to detect *gfp* transcripts, as this is a more sensitive method than detecting fluorescence of the protein.

Electrophoretic gel mobility shift analysis

Embryos were collected and the region of the cement gland primordium, including both ectodermal layers and some underlying endoderm, dissected at stages 15-17. Explants were homogenized (4 μl/explant) in 50 mM Tris, 50 mM KCl, 2 mM DTT, 1 mM EDTA, 20% glycerol, 1× protease inhibitors (Complete Tablet without EDTA; Roche), 1 mM NaF, 10 mM β-glycerophosphate. Probe was made by annealing top and bottom strand oligos and filling in with Klenow in the presence of ^{32}P-dGTP or dCTP. Binding was carried out in 36 mM Tris pH 8, 18 mM KCl, 1.4 mM DTT, 3.6 mM $MgCl_2$, 0.7 mM EDTA, 1 mM NaF, 10 mM β-glycerophosphate, 1×protease inhibitors (as above) and 300 ng/μl poly (dI-dC; Amersham) with 4 μl extract and 10,000 cpm probe with or without 200×cold competitor.

Microinjection and RT-PCR

Embryos were collected and dejellied as described (Sive et al., 2000). Embryos were injected at the one- to two-cell stage with 50 pg of plasmid DNA as indicated in the text and 150 pg of *globin* or *otx2* mRNA. Alternatively, the transgenesis protocol above was followed with 5×EBS, 5×CRE and TATA only constructs, embryos were sorted at the two-cell stage and injected with 150 pg of *globin* or *otx2* mRNA, or left uninjected. Animal caps were cut at stage 9 from injected embryos and cultured to stage 17-20. RNA and cDNA from pools of 15-25 caps was prepared as described (Kolm and Sive, 1995). The uninjected embryos were left to develop to tailbud stage, then processed for in situ to check the efficiency of transgenesis. Primers used were XCG (17 cycles), XAG (19 cycles) and ODC (21 cycles) as described elsewhere (Gammill and Sive, 1997; Sun et al., 1999). For GFP, 22 cycles were used with the following primers: GFP.L (listed above) and GFP.U (5′ ACATCATGGCAGACAAACCA).

Sequence analysis

Potential transcription factor binding sites in the *Xag1* promoter were identified using MatInspector V2.2 (Quandt et al., 1995).

RESULTS

Characterization of the *Xag1* upstream region

In order to define the transcriptional start site of the *Xag1* transcript, we performed 5′ RACE on *Xag1* mRNA. This analysis yielded two classes of transcripts. The first class begins at an initiator region 25 bp downstream of a TATA box. The second class consists of multiple transcripts, each of slightly different length, which extend into a 50 bp region 100-150 bp upstream of the TATA box (see Fig. 1A,C). Northern analysis using probes designed to hybridize to the 5′ end of the different transcript types (probes 1 and 2, Fig. 1A), shows that the first class of transcript, which initiates downstream of the TATA box, is the most abundant by at least 30-fold in the embryo. Given these data, we began to test ability of this TATA-containing region to drive reporter gene expression in the cement gland.

8 kb of genomic *Xag1* sequence drives expression of a reporter gene in a pattern indistinguishable from endogenous *Xag1*

As a first step in analyzing regulation of *Xag1* transcription, a reporter gene nuclear green fluorescent protein (nGFP),

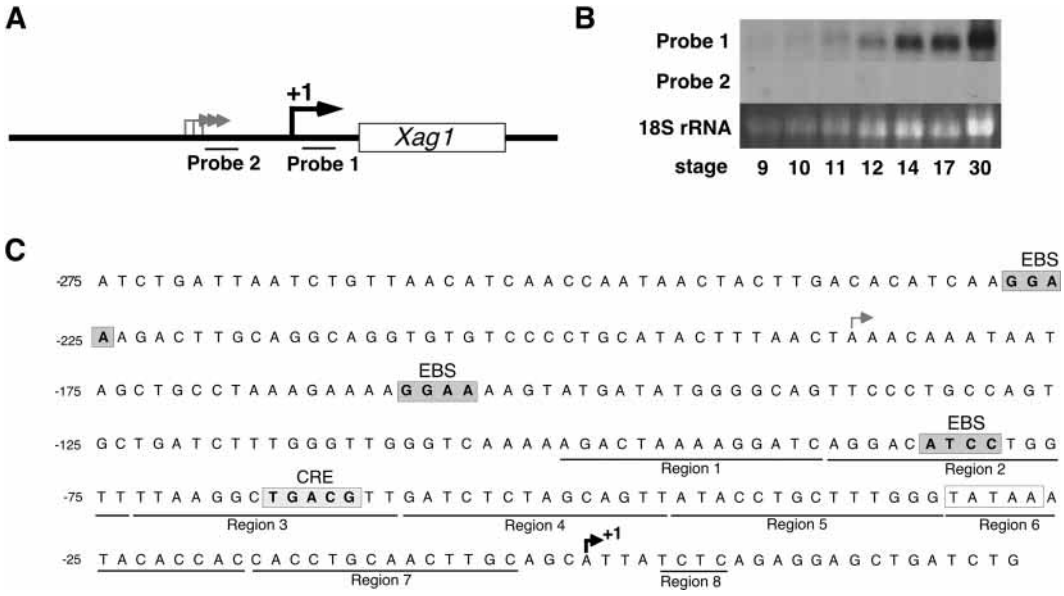

Fig. 1. (A) Two major classes of *Xag1* transcripts are detected by 5′ RACE. One class initiates downstream of a TATA box (+1; black arrow). The second class consists of several transcripts that initiate 150-100 bp upstream of the TATA box (gray arrows). (B) Northern blot analysis of RNA isolated from blastula through tailbud (stage 9-30) embryos shows the first class of transcript is the most abundant in the embryo. Both probes recognize in vitro transcribed *Xag1* upstream sequence (not shown). (C) Sequence of the *Xag1* genomic region used in this study. 3 Ets-like binding sites (EBS; dark-gray boxes), an ATF/CREB-like binding site (CRE, light-gray box) and the TATA box (unshaded box) are indicated. The black arrow indicates the initiation site of the major transcript, the gray arrow indicates the 5′ limit of the rarer transcripts that were isolated. Also shown are the 14 bp regions (1-7) and the 4 bp region 8 that were replaced in the linkerscan analysis (see Fig. 3).

was inserted into the 5′ UTR of the *Xag1* genomic locus that contains upstream sequence, protein coding exons and introns (see Materials and Methods). Transgenic *X. laevis* embryos were generated with this construct and analyzed for *gfp* expression either by GFP fluorescence or in situ hybridization for the *gfp* transcript. Twenty-nine percent of the embryos generated express *gfp* in both the cement and hatching gland primordia from the end of gastrulation until tadpole stages (Fig. 2C,D) in a pattern indistinguishable from endogenous *Xag1* (Fig. 2A,B). This percentage of transgenic embryos expressing GFP is typical of many active promoters (Kroll and Amaya, 1996; Sparrow et al., 2000; Davis et al., 2001). This observation indicates that 8 kb of genomic upstream sequence contains all the information required to drive expression of transcripts in the endogenous *Xag1* pattern.

102 bp of genomic sequence upstream of the transcription start site is sufficient to drive cement gland-specific expression

In order to narrow down the sequences responsible for cement gland-specific expression, transgenic embryos were generated with deletion constructs containing between 275 bp and 73 bp of genomic sequence upstream of the transcription start site (see Materials and Methods; Fig. 1). Constructs containing 102 bp or more upstream of the transcription start site are sufficient to drive *gfp* expression specifically to the cement gland from the end of gastrulation in 30-59% of embryos generated (Figs 2 and 3;

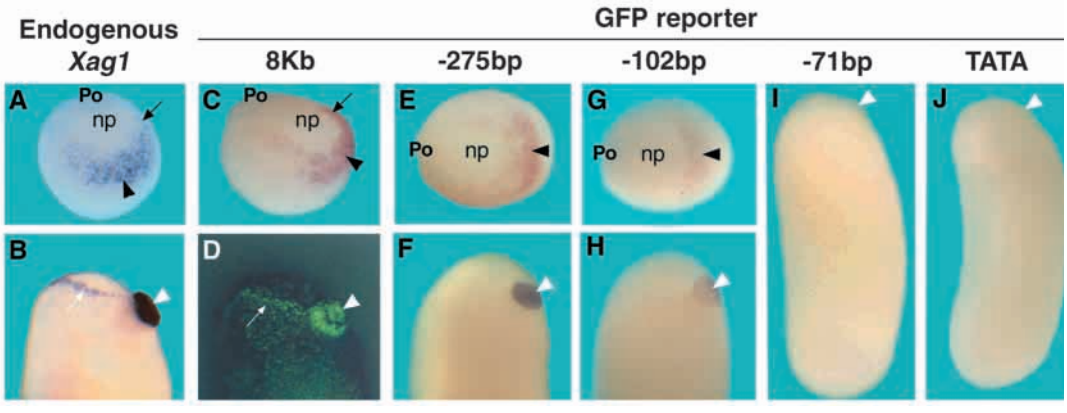

Fig. 2. Upstream genomic *Xag1* sequence drives cement gland-specific expression. In situ hybridization for endogenous *Xag1* (A,B) or *gfp* transcripts (C,E-J) in early neurula (A,C,E,G) or tailbud (B,F,H-J) embryos. (D) GFP fluorescence in a transgenic tailbud embryo. Endogenous *Xag1* is expressed at the anterior of the embryo from the end of gastrulation (A) in the cement gland (black arrowhead) and hatching gland primordia (black arrow), and in the cement gland (white arrowhead) and hatching gland (white arrow) in the tailbud embryo (B). This expression pattern is replicated by 8 kb of *Xag1* genomic sequence driving a GFP reporter gene (C,D). (E-H) Cement gland-specific *gfp* expression is driven from early neurula stages by –275 bp (E,F) and –102 bp (G,H), but not 73 bp of upstream sequence (I) or the *Xag1* TATA box (J) (arrowheads indicate location of cement gland). Expression is weaker with the –102 bp construct (G,H) than with the longer –275 bp construct (E,H), especially at neurula stages (arrowheads). Po, posterior; np, neural plate.

Table 1). In these embryos, *gfp* expression in the cement gland is confined to the outer layer of ectoderm (data not shown) and ectopic expression in other parts of the embryo is not seen, nor is expression seen in the hatching gland. Although the in situ protocol is not quantitative, the –102 bp construct shows consistently less intense *gfp* expression than the longer –161 bp or –275 bp deletion constructs (Fig. 2, compare E and F with G and H), even though frequency of expression is similar for all of these constructs (Table 1). Seventy-three base pairs of upstream sequence are not sufficient to drive reporter gene expression, except in 1% of cases (Table 1). A construct containing only the TATA box region (–20 to +23 bp) driving *gfp* is also insufficient to drive expression (Table 1). As already mentioned, *Xag1* is also expressed in the hatching gland (Fig. 2), and later during tadpole stages in the developing lung buds (L. Bradley and H. L. S, unpublished). Eight kilobases of genomic *Xag1* sequence is able to recapitulate hatching gland and lung expression, but the short, –102 bp *Xag1* promoter does not (Fig. 2 and data not shown), confirming that the sequences present in this region are specific for cement gland expression.

Multiple sites in the minimal promoter are required for expression

In order to identify regions of the –102 bp minimal promoter sequence required for cement gland-specific expression, a series of linkerscan replacements were made between –102 bp and +8 bp (see Materials and Methods; Figs 1 and 3). Transgenic embryos were generated with these constructs and scored for *gfp* expression at early tailbud stages (stage 20-26). The results indicate that several regions within this short piece of genomic sequence are important for transcription. First, embryos with a linkerscan replacement in region 2, covering a putative Ets-like binding site (EBS; GGAA/T) (reviewed by Sharrocks, 2001) do not express *gfp* in the cement gland, except in 1% of cases (Table 1). This is consistent with the observation above that deleting the region between –102 bp and –73 bp, which contains the EBS, almost completely abolishes expression. Additional regions important for robust *gfp* expression include region 3, which contains a putative cAMP-responsive element (CRE) half site (TGACG) (Fink et al., 1988; Paca-Uccaralertkun, 1994), region 6, which contains the TATA box, and regions 1 and 5, which are not predicted to contain known transcription factor binding sites (Fig. 3; Table 1). Region 4 and region 7, which may contain an SP1 binding site, are also important for *gfp* expression, although to a lesser extent than the regions already mentioned. In summary, most of the 102 bp upstream of the transcription start site is important for cement gland-specific expression.

Sequences between –275 bp and –102 bp enhance expression

In addition to the linkerscan replacements described, the same

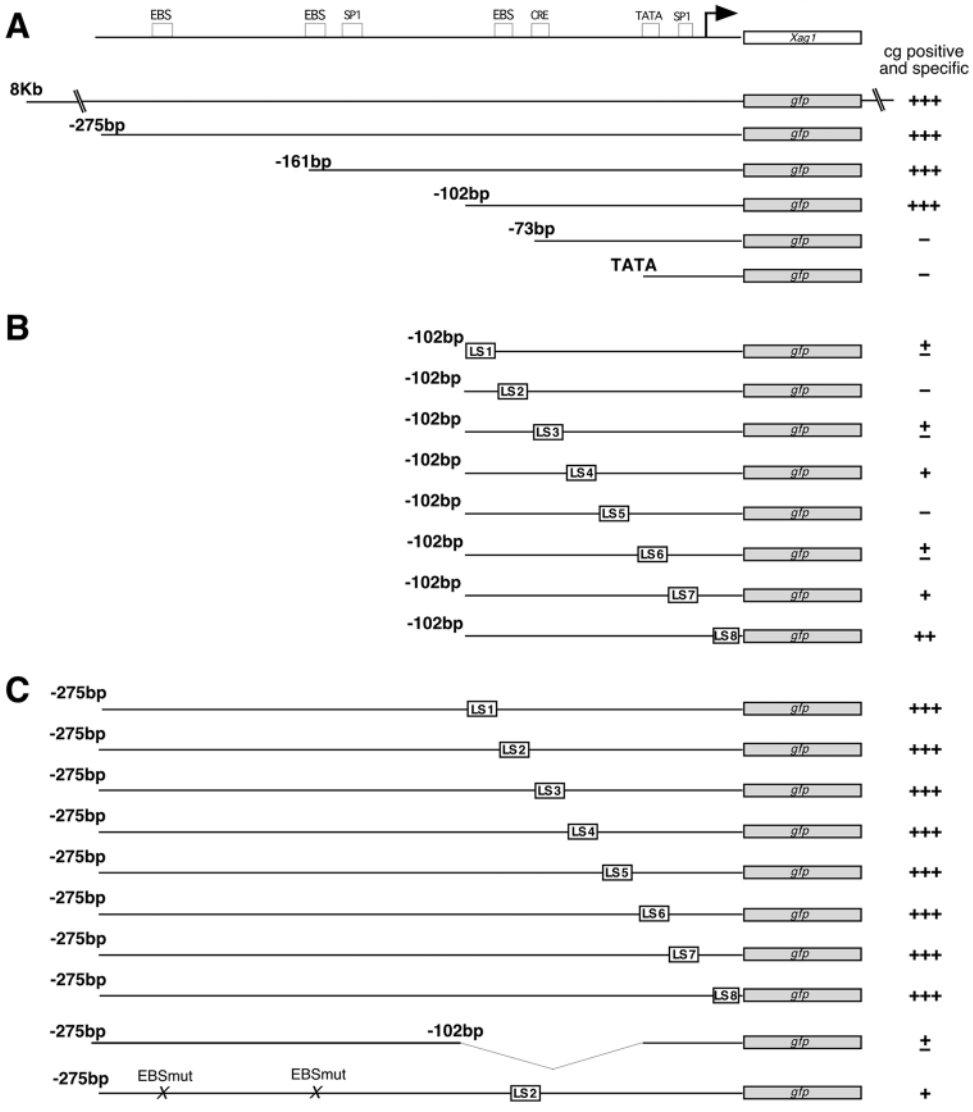

Fig. 3. Structure of *Xag1* upstream region and reporter constructs. (A) Potential transcription factor-binding sites and the start site of transcription (black arrow) are indicated. Cement gland-specific expression is driven at tailbud stages by –102 bp of upstream sequence. For the 8 kb construct, hatching gland expression is also seen. (B) Linkerscan analysis of this short promoter shows linkerscan (LS) regions 1-3 and 5-6 are most important for this expression. (C) In the context of the larger –275 bp promoter, mutation of these regions has no effect on *gfp* expression, although the distal promoter alone drives expression only very rarely. A large part of the distal compensation can be attributed to the two EBS found in this region, because when these are mutated the frequency of expression in the cement gland falls significantly. Cement gland expression of *gfp* in more than 25% of embryos is indicated by +++ (18-25%), ++ (9-17%), + (2-8%), +/– (no change) and – (<2%) (see Table 1; Materials and Methods).

Table 1. Deletion and linkerscan analysis of the *Xag1* promoter

Construct*	Total number embryos scored†	Number with cement gland-specific expression‡	% with cement gland-specific expression
8 kb§	112	33	29
–275 bp	137	81	59
–161 bp	84	40	48
–102 bp	217	65	30
–73 bp	166	2	1
TATA	95	0	0
–102 bp linkerscans			
LS1	143	8	5
LS2	168	2	1
LS3	166	5	3
LS4	119	11	9
LS5	97	2	2
LS6	125	5	4
LS7	107	13	12
LS8	149	37	25
–275 bp linkerscans			
LS1	65	27	42
LS2	161	61	38
LS3	111	53	48
LS4	159	68	43
LS5	94	35	37
LS6	35	17	49
LS7	55	15	27
LS8	60	22	37
–275/–102 bp	118	4	3
–275LS2.EBSmut	89	12	13

*Constructs contain the *Xag1* TATA box and transcription initiation region. Reporter gene in all constructs was *gfp*. See Fig. 3 for construct design.
†From at least two independent experiments.
‡Embryos were scored for *gfp* expression in the cement gland by in situ hybridization. Because the in situ protocol is not quantitative, embryos were scored on the basis of presence or absence of *gfp* expression, and intensity of staining was not taken into account.
§Hatching gland expression is also seen with the 8 kb construct.

Table 2. Sufficiency of *Xag1* promoter regions to drive cement gland-specific *gfp* expression

Construct*	Total number embryos scored†	Number with cement gland-specific expression‡	% with cement gland-specific expression
5xreg1	59	0	0
5EBS	250	45	18
5CRE	214	39	18
5xreg5	118	0	0
5EBS.mut	112	1	1
5CRE.mut	148	2	1
3EBS.2CRE	77	20	26
MLP	106	0	0
5EBS.MLP	100	2	2
5CRE.MLP	110	5	4
3EBS.2CRE.MLP	52	18	35

*Constructs contain the *Xag1* TATA box and transcription initiation region, except those indicted by MLP, which contain the adenovirus major late promoter. Reporter gene in all constructs was *gfp*. See Fig. 4 for construct design.
†From at least two independent experiments
‡Embryos were scored for *gfp* expression in the cement gland by in situ hybridization. Because the in situ protocol is not quantitative, embryos were scored on the basis of presence or absence of *gfp* expression, and intensity of staining was not taken into account.

replacements were tested in the context of the longer, –275 bp, promoter. Embryos transgenic for these constructs were scored at early tailbud stages and found to express *gfp* at the same frequency and levels as the wild type –275 bp construct, suggesting that sites in the more distal promoter compensate for the loss of sites in the proximal promoter, including the TATA box. To test whether the distal region alone is sufficient to drive expression of *gfp* to the cement gland, we made a construct consisting of the region from –275 bp to –102 bp placed in front of the *Xag1* TATA box. This construct is poor at driving transcription, with cement gland-specific expression seen in only 3% of embryos (Table 1), indicating that although sites in the distal region enhance expression they are not sufficient to drive cement gland expression. We noticed two further EBS present in the distal promoter region (Fig. 1). To test whether the enhancing activity of the distal promoter can be attributed to these, we mutated the two EBS (GGAA to aGgc) in the –275 bp construct that also has a linkerscan replacement covering the proximal EBS (region 2), so that all three EBS were mutated. Embryos transgenic for this construct show cement gland-specific expression of *gfp* in 13% of cases (Table 1), a substantial decrease when compared with the construct containing only the proximal EBS replacement (38%; Table 1). These data confirm that a large part of the compensation shown by the distal promoter can be attributed to the two distal Ets-binding sites.

The EBS and CRE are sufficient to drive cement gland-specific expression

As the EBS, CRE and regions 1 and 5 were found to be important for cement gland-specific expression, we next asked whether they are sufficient for expression. Each region was individually multimerized fivefold and subcloned in front of the *Xag1* TATA box region (–20 to +23 bp). Multimerized EBS or CRE elements (5xEBS and 5xCRE) drive cement gland-specific expression of *gfp* at early tailbud stages in 18% of cases (Table 2; Fig. 4), ectopic expression was not seen and sectioning confirmed that expression was limited to the outer layer of ectoderm (not shown). Multimerized regions 1 or 5, however, do not drive detectable reporter gene expression (Table 2; Fig. 4). *gfp* expression with 5xEBS and 5xCRE was also assayed at early neurula stages; however, we were not able to detect expression until late neurula stages (stage 18). This may be because expression driven by the 5xEBS and 5xCRE constructs is very weak at early stages and so we were unable to detect it by in situ hybridization, or that these constructs are not able to drive very early expression. Mutating the core recognition sequences in 5xEBS (GGAT to aGgc; 5xEBSmut) and in 5xCRE (TGACGT to TGtgGc; 5xCREmut) causes almost complete loss of *gfp* expression, except 1% of cases, confirming the importance of these binding sites for expression.

The EBS and CRE cooperate to drive cement gland-specific gene expression

Ets-related proteins generally interact with other transcription factors to regulate gene expression (Li et al., 2000). The

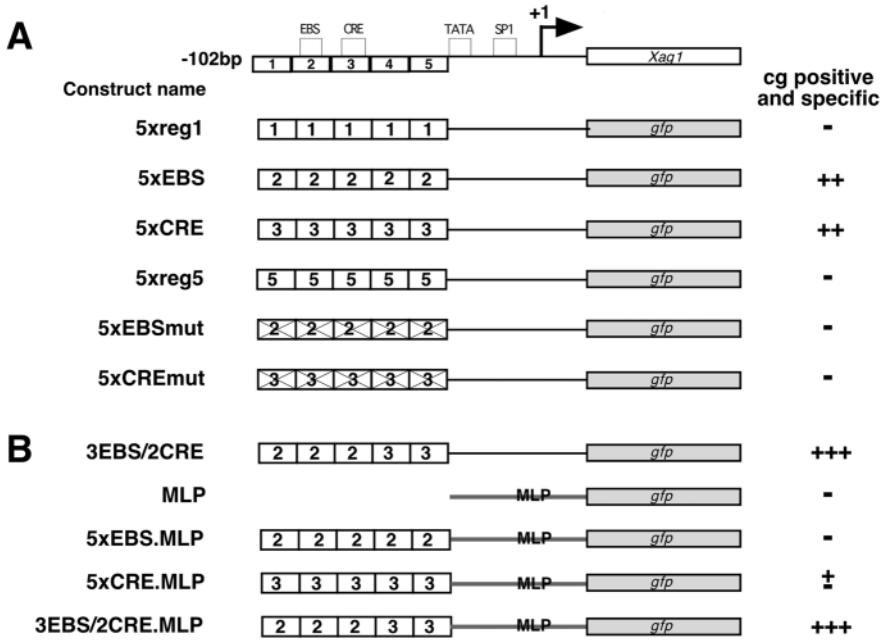

Fig. 4. The EBS and CRE are sufficient to drive cement gland expression in the tailbud embryo and cooperate to increase expression. (A) Multimerized 14 bp regions containing the proximal EBS (region 2) or CRE (region 3) are sufficient to drive cement gland-specific expression of GFP. When the EBS or CRE are mutated (3 bp substitution in core binding site; see Materials and Methods), their ability to drive this expression is abolished. (B) In front of a heterologous promoter (adenovirus major late promoter; MLP) the 5xEBS and 5xCRE are extremely inefficient at driving *gfp* expression, but when multimerized together (3EBS/2CRE) the sites cooperate to drive robust expression in the cement gland. Cement gland expression of *gfp* in more than 25% of embryos is indicated by +++ (18-25%), ++ (9-17%), + (2-8%), +/– (no change) and – (<2%) (see Table 2; Materials and Methods).

proximity of the EBS and CRE suggested to us that these two sites may cooperate to drive expression. To address this, we compared the ability of the 5xEBS or 5xCRE constructs to drive cement gland-specific *gfp* expression at early tailbud stages with a construct containing five binding sites in the combination three EBS and two CRE (3EBS/2CRE). The 3EBS/2CRE construct drives cement gland-specific expression at slightly increased frequencies compared with 5xEBS or 5xCRE (26% compared to 18%; Table 2) when in front of the *Xag1* TATA box. This cooperation is more pronounced, however, if a heterologous promoter (the adenovirus major late promoter; MLP) replaces the *Xag1* TATA box and downstream sequence. In this case, the 5xEBS.MLP or 5xCRE.MLP constructs drive expression to barely more than background levels (2% and 4%; Table 2). However the 3EBS/2CRE.MLP construct gives cement-gland specific expression at a frequency (35%; Table 2) similar to whole promoter constructs (29-59%; Table 1). As before, no ectopic expression was seen and we were unable to detect *gfp* expression at early neurula stages, although robust expression was observed starting at late neurula (stage 18). These results show that the EBS and CRE functionally cooperate, and also suggest that the EBS or CRE sites individually cooperate with the *Xag1* TATA box, but not the MLP, region drive robust expression.

Xag1 promoter binding activities are present in early neurula stage cement gland

To ask whether specific binding activities are present in the neurula stage embryo, electrophoretic gel mobility shift assays were performed with whole cell extracts from cement gland regions (ectoderm plus underlying endoderm) isolated from mid-neurula (stage 15-17). Probe corresponded to regions 1-5 of the *Xag1* promoter, including the EBS and CRE. Fig. 5 shows that an activity binding the –102 bp promoter region (lane 2) is competed by cold *Xag1* competitor (lane 3), but not a probe for the OCTA binding site, which acts as a nonspecific control (lane 8) (Hinkley and Perry, 1991). This activity is also competed by a cold competitor for the wild-type EBS (lane 4) but not a mutated EBS (lane 5). In addition, the binding complex is competed by a cold competitor for the wild-type CRE, although competition is less strong than the EBS probe. The mutated CRE site, which abrogates promoter activity in the analysis above, weakly competes for binding under these conditions. A cold probe corresponding to regions 4 and 5 does not compete for complex binding (not shown). These results suggest that the gel shift activity observed may consist of a complex containing both an EBS-binding factor and a CRE-binding factor. At this time, we do not know whether these binding activities are specific for the cement gland.

The CRE but not the EBS responds to Otx2 activity

Otx2 indirectly induces *Xag1* expression (Gammill and Sive, 1997), indicating that intermediary factors are required for Otx2 action. Factors binding the EBS and CRE in the *Xag1* promoter may act downstream of Otx2, or may lie in an independent pathway. In order to test whether the EBS and CRE respond to Otx2, we performed an ectodermal explant (animal cap) assay. Embryos were injected with plasmid DNA for either 5xEBS, 5xEBSmut, 5xCRE, 5xCREmut, 3EBS/2CRE or TATA-only nGFP constructs (Fig. 6) along with either *globin* control mRNA or *otx2* mRNA. Animal caps were cut at stage 9 and cultured to mid-neurula stages when they were collected and analyzed by RT-PCR for expression of the cement gland markers, *Xag1* and *Xcg*, and for *gfp* expression. As expected, injection of *globin* mRNA does not induce cement gland fate or *gfp* expression. (Fig. 6, lanes 2-7). Injection of *otx2* mRNA induces both *Xag1* and *Xcg1* expression in caps (Fig. 6, lanes 8-13). *otx2* mRNA injection also induces *gfp* expression in caps injected with the 5xCRE or 3EBS/2CRE construct (lanes 9 and 12), but not those injected with the TATA only (lane 7), 5xEBS (lane 8), mutated EBS (lane 10) or mutated CRE (lane 11) constructs. Similar results were obtained with caps isolated from embryos transgenic for EBS and CRE constructs, and injected with *otx2*

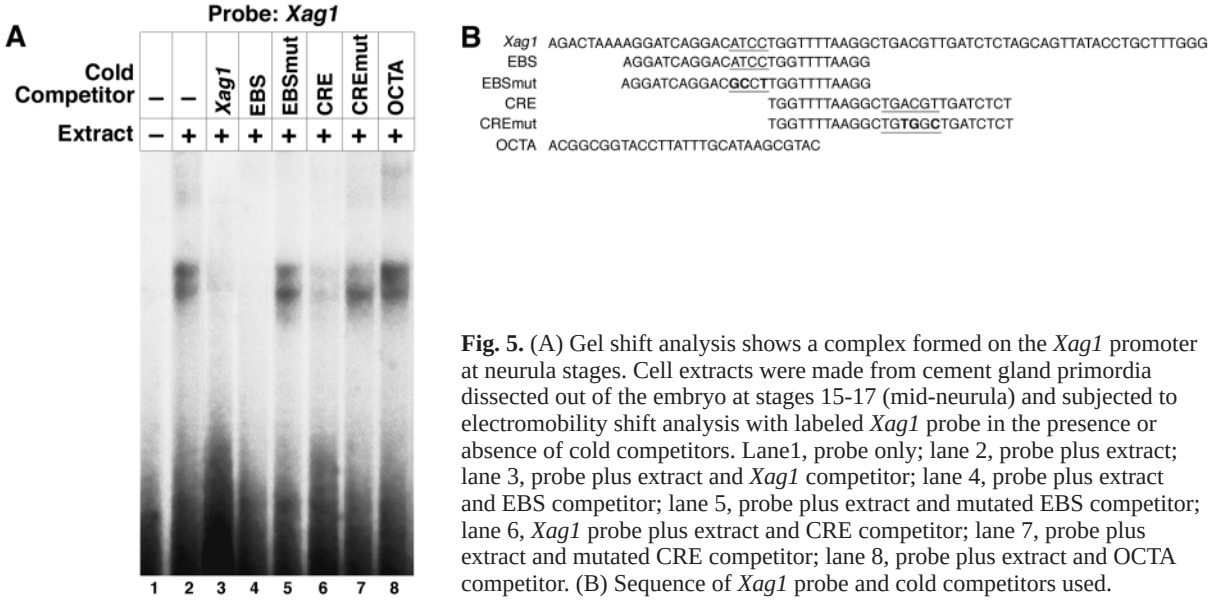

Fig. 5. (A) Gel shift analysis shows a complex formed on the *Xag1* promoter at neurula stages. Cell extracts were made from cement gland primordia dissected out of the embryo at stages 15-17 (mid-neurula) and subjected to electromobility shift analysis with labeled *Xag1* probe in the presence or absence of cold competitors. Lane1, probe only; lane 2, probe plus extract; lane 3, probe plus extract and *Xag1* competitor; lane 4, probe plus extract and EBS competitor; lane 5, probe plus extract and mutated EBS competitor; lane 6, *Xag1* probe plus extract and CRE competitor; lane 7, probe plus extract and mutated CRE competitor; lane 8, probe plus extract and OCTA competitor. (B) Sequence of *Xag1* probe and cold competitors used.

mRNA. These results suggest that two pathways regulate the expression of *Xag1*. One pathway involves Otx2, mediated by the CRE in the *Xag1* promoter, the other pathway is independent of Otx2 and is mediated by the EBS.

DISCUSSION

We have asked how the *Xenopus* cement gland is positioned at the extreme anterior of the embryo, by analyzing how the promoter of the *Xag1* gene, a marker of cement gland differentiation, is activated. We show that members of the Ets and ATF/CREB transcription factor families are likely to integrate positional information that determines the cement gland and activates *Xag1* expression specifically in the cement gland.

Ets- and ATF/CREB-like binding sites cooperate to activate *Xag1* expression

Ets-binding sites (EBS; GGAA/T) interact with members of a family of transcriptional regulators that share a conserved Ets domain (reviewed by Sharrocks, 2001). cAMP-responsive elements (CRE; TGACG) interact with both CREBs and ATFs, which belong to a large family of transcriptional regulators containing a conserved bZip domain (reviewed by Hai and Hartman, 2001). The proximal EBS and CRE in the *Xag1* promoter are both necessary and sufficient for cement gland-specific expression of *Xag1*, as mutation of either site in the context of the short, –102 bp promoter causes a severe decrease

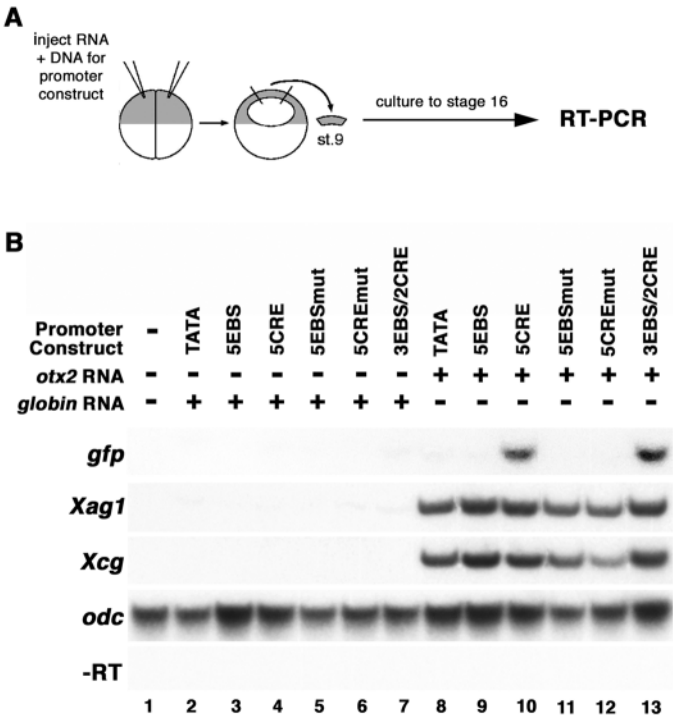

Fig. 6. The CRE responds to Otx2 activity, while the EBS is independent of Otx2. (A) Embryos were injected at the 1- to 2-cell stage with 150 pg *otx2* or *globin* mRNA plus 50 pg of either 5xEBS, 5xEBSmut, 5xCRE, 5xCREmut, 3EBS/2CRE or TATA-only nGFP constructs. Promoter constructs contained the *Xag1* TATA box (see Fig. 4). Animal caps were cut at stage 9, cultured until stage 16 and expression of *Xcg*, *Xag1*, *gfp* and *odc* analyzed by RT-PCR. (B) RT-PCR of ectodermal explants dissected from embryos injected with *otx2* or *globin* mRNA plus the indicated reporter construct. Expression of cement gland markers (*Xcg* and *Xag1*) and induced *gfp* were examined using *odc* expression as a loading control. Lane 1, uninjected animal caps; lane 2, *globin* mRNA plus TATA-only nGFP DNA; lane 3, *globin* mRNA plus 5xEBS.nGFP DNA; lane 4, *globin* mRNA plus 5xCRE.nGFP DNA; lane 5, *globin* mRNA plus 5xEBSmut.nGFP DNA; lane 6, *globin* mRNA plus 5xCREmut.nGFP DNA; lane 7, *globin* mRNA plus 3EBS/2CRE.nGFP DNA; lane 8, *otx2* mRNA plus TATA-only nGFP DNA; lane 9, *otx2* mRNA plus 5xEBS.nGFP DNA; lane 10, *otx2* mRNA plus 5xCRE.nGFP DNA; lane 11, *otx2* mRNA plus 5xEBSmut.nGFP DNA; lane 12, *otx2* mRNA plus 5xCREmut.nGFP DNA; lane 13, *otx2* mRNA plus 3EBS/2CRE.nGFP DNA.

in expression, while multimerized sites are able to drive expression (Figs 3, 4). The longer –275 bp promoter contains three EBS, and while deletion of the proximal EBS in this construct has no effect on promoter activity, mutation of all three sites severely depresses promoter activity, further indicating the importance of this class of binding site. In the context of a heterologous promoter, the EBS and CRE co-operate, and are also likely to do so in the intact promoter. Physical and functional interaction of Ets and ATF/CREB factors has been demonstrated in several other systems (Giese et al., 1995; Papoutsopoulou and Janknecht, 2000).

Several Ets factors have been identified in *Xenopus* (Baltzinger et al., 1999; Chen et al., 1999; Münchberg and Steinbeisser, 1999; Meyer et al., 1997; Meyer et al., 1995; Gorgoni et al., 1995); however, none is expressed in the cement gland primordium or modulates cement gland formation (Goltzené et al., 2000; Remy et al., 1996). A CRE-binding activity has been identified in *Xenopus* embryos (Lutz et al., 1999), and a dominant-negative CREB construct causes microcephaly, although cement glands are able to form in these embryos. *Xenopus* Jun, another bZip protein that can interact with the CRE, promotes ventral development when misexpressed (Knochel et al., 2000); however, it is not clear whether Jun plays any role in cement gland formation.

Multiple sites in the *Xag1* promoter are likely to cooperate

Although the EBS and CRE together provide sufficient information to drive cement gland-specific reporter gene expression, other sites in the *Xag1* promoter are likely to co-operate to drive robust expression. In particular, the longer, –275 bp, promoter appeared to give stronger reporter expression than the shorter, –102 bp, region. However, the distal region (–275 to –102 bp), placed in front of the *Xag1* TATA box, cannot substitute for the region downstream of –102 bp (Fig. 3). In addition to the EBS and CRE, three other regions in the short promoter are important for reporter gene expression, including the TATA box and two regions that do not appear to contain binding sites for known transcription factor families. Two transcription factors that may act downstream of Otx2 to regulate *Xag1* expression include *pitx1* and *pitx2c*, paired-class homeodomain proteins that are expressed in both the cement gland and stomodeal primordia. Ectopic expression of these genes can activate cement gland formation (Hollemann and Pieler, 1999; Chang et al., 2001; Schweikert et al., 2001). Interestingly, we find no evidence for *pitx*-binding sites in the *Xag1* promoter, indicating that regulation of *Xag1* by these factors is indirect. The importance of these other sites in the context of the whole promoter is underscored by the inability of multimerized EBS or CRE alone constructs to drive reporter gene expression from a heterologous promoter (Fig. 4). Together, the data suggests that multiple co-operating factors regulate *Xag1* promoter function.

Restricting *Xag1* expression to the cement gland

Xag1 expression could be restricted to the cement gland through positively acting factors alone, with expression or activity of these factors limited to the cement gland primordium. In support of this, we have found no evidence for a distinct repressor region in the *Xag1* promoter, which when removed leads to ectopic reporter gene expression. However, putative Ets and ATF/CREB factors, which interact with the EBS or CRE and act positively in the cement gland, could be inactivated or converted into repressors outside this region by post-translational modification (Mayr and Montminy, 2001; Sharrocks, 2001). Additionally, different Ets and ATF/CREB proteins can act as activators and repressors, by binding to the same DNA element with opposing outcomes (Rebay and Rubin, 1995; O'Neill et al., 1994). It is therefore possible that these classes of factor both activate *Xag1* expression in the cement gland and repress its expression elsewhere.

Integration of Otx2-dependent CRE activity and Otx2-independent EBS activity

Current data suggests that formation of the cement gland requires integration of anterodorsal (AD), ventrolateral (VL) and outer layer ectodermal (EO) domains. Which domains might regulate putative Ets and ATF/CREB factors that interact with the *Xag1* promoter? Our data show that the CRE present in the *Xag1* promoter is activated by Otx2, indicating that it lies downstream of Otx2 and is a readout of the AD domain (Fig. 7).

By contrast, the inability of Otx2 to activate the EBS suggests that a factor binding to this site acts in an Otx2-independent pathway. Although the EBS is crucial for cement gland-specific gene expression, it is only sufficient to direct this expression in combination with either the *Xag1* TATA box region or the CRE. This suggests that a factor binding to the EBS interacts with an Otx2-dependent factor(s) that binds either to the CRE or to the *Xag1* TATA box region. We suggest this because the ADMLP cannot substitute for the *Xag1* TATA region, suggesting that this region responds to anterior

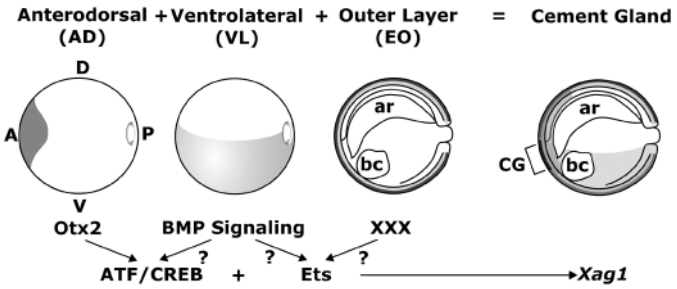

Fig. 7. Model to show how transcription factor activity is integrated at the *Xag1* promoter to bring about cement gland-specific expression. Markers of differentiation, such as *Xag1*, are activated at the end of gastrulation in the cement gland primordium (CG), which is defined by the overlap of up to three larger domains: anterodorsal (AD), ventrolateral (VL) and ectodermal outer layer (EO). Surface views of early neurula stage embryos are shown for the AD and VL panels, and sagittal section schematics for the EO and cement gland panels. The AD domain is defined by *otx2* expression, the VL domain by BMP4 or some downstream readout, such as activated Smad1, while an unknown factor (XXX) defines the outer ectodermal layer. *Xag1* expression in the cement gland primordium requires an ATF/CREB factor, which binds to the CRE and lies downstream of *otx2*. This acts in cooperation with an Ets factor that binds to the EBS and may lie downstream of BMP4 and/or XXX. Other inputs may influence *Xag1* expression, but are not sufficient on their own to drive expression. CG, cement gland; ar, archenteron; Bc, blastocoel; A, anterior; P, posterior; D, dorsal; V, ventral.

positional information. In both cases, this factor would be a readout of the AD domain but not sufficient to drive cement gland-specific gene expression. Alternatively, it is possible that an anterior-specific factor distinct from Otx2 activates the EBS-binding factor, which would, in fact, represent a readout of the AD domain (Fig. 7).

Although cement gland positioning requires interaction of three domains, two factor-binding sites (the EBS and CRE together or singly in combination with the *Xag1* TATA box region) are sufficient for cement gland-specific reporter expression. This suggests that one or both of these sites must integrate the readout of more than one domain. This integration could represent an intermediate step in cement gland positioning, e.g. an extreme anteriodorsal domain, which is not germ layer specific, defined by AD+VL. This predicts that reporter gene activation is observed in the relevant domain from an appropriate construct. The lack of any reporter gene readout in such intermediate domains may reflect the absence of stable promoter binding by either factor alone.

In order to characterize the domains in which *Xag1* regulatory factors act, and to further understand how positional information is integrated to direct cement gland-specific gene expression, we are currently identifying candidate factors that interact with the EBS and CRE in the *Xag1* promoter. We are additionally asking whether these classes of factor are used by other cement gland differentiation genes.

We acknowledge Brenda Kennedy for attempting *Xag1* promoter analysis long ago, and Dave Willison for sequencing an *Xag1* genomic clone. We thank Annemarie Schoen for help with Fig. 7. We thank Vladimir Apekin for expert frog care, and members of our laboratory for critical reading of the manuscript. This work was supported by a grant from the NSF to H. L. S. (IBN-9876393). F. C. W. was a Herman and Margaret Sokol Fellow. D. H. W. was a HHMI pre-doctoral fellow.

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