Misexpression of chick Vg1 in the marginal zone induces primitive streak formation

Shailan B. Shah[1], Isaac Skromne[2], Clifford R. Hume[1,*], Daniel S. Kessler[3], Kevin J. Lee[4], Claudio D. Stern[2] and Jane Dodd[1,†]

[1]Department of Physiology and Cellular Biophysics, [2]Department of Genetics and Development, [4]Howard Hughes Medical Institute, [1,2,4]Center for Neurobiology and Behavior, College of Physicians and Surgeons of Columbia University, 630 West 168th Street, New York, NY 10032, USA
[3]Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA
*Current address: Virginia Merrill Bloedel Hearing Research Center, University of Washington, Seatt;e. WA98195, USA

[†]Author for correspondence (e-mail: jd18@columbia.edu)

SUMMARY

In the chick embryo, the primitive streak is the first axial structure to develop. The initiation of primitive streak formation in the posterior area pellucida is influenced by the adjacent posterior marginal zone (PMZ). We show here that chick *Vg1 (cVg1),* a member of the TGFβ family of signalling molecules whose homolog in *Xenopus* is implicated in mesoderm induction, is expressed in the PMZ of prestreak embryos. Ectopic expression of cVg1 protein in the marginal zone chick blastoderms directs the formation of a secondary primitive streak, which subsequently develops into an ectopic embryo. We have used cell marking techniques to show that cells that contribute to the

ectopic primitive streak change fate, acquiring two distinct properties of primitive streak cells, defined by gene expression and cell movements. Furthermore, naive epiblast explants exposed to cVg1 protein in vitro acquire axial mesodermal properties. Together, these results show that cVg1 can mediate ectopic axis formation in the chick by inducing new cell fates and they permit the analysis of distinct events that occur during primitive streak formation.

Key words: cVg1, primitive streak, axis formation, induction, chick, TGFβ, *Vg1*

INTRODUCTION

The primitive streak is the first axial structure to develop in the chick blastoderm. All embryonic mesoderm and endoderm derive from the primitive streak. However, the molecular and cellular events underlying primitive streak formation remain poorly defined. Prior to primitive streak formation, the chick blastoderm is a bilaminar disc composed of an epiblast layer, which gives rise to all embryonic tissues, and a hypoblast layer, which contributes to extraembryonic structures. The epiblast consists of a central disc (the area pellucida), the narrow marginal zone and the outer area opaca. Epiblast cells of the area pellucida migrate towards the posterior marginal zone (PMZ) and then coalesce to form the primitive streak (Spratt and Haas, 1965; Vakaet, 1970, 1984; Hatada and Stern, 1994).

Classical embryological experiments have shown that the PMZ plays an important role in primitive streak formation (Spratt and Haas, 1960; Khaner and Eyal-Giladi, 1986, 1989). Transplantation of the PMZ to a lateral position results in the formation of an ectopic primitive streak in the area pellucida epiblast adjacent to the graft site (Khaner and Eyal-Giladi, 1986, 1989; Eyal-Giladi and Khaner, 1989).

The signals involved in primitive streak formation have not been identified. Recently, several molecules have been described that can initiate axial development in *Xenopus*, including the TGFβ family members, activin (Smith et al.,

1990), Vg1 (Thomsen and Melton, 1993) and nodal-related (Ecochard et al., 1995; Jones et al., 1995; Smith et al., 1995). Of these, only Vg1 is expressed in a temporal and spatial pattern consistent with an endogenous role in *Xenopus* axis formation (Weeks and Melton, 1987; Dohrmann et al., 1993).

TGFβ family members have also been proposed to play a role in mesoderm formation in the chick embryo. Activin supports the development of axial structures from epiblast isolated from the hypoblast and marginal zone (Mitrani and Shimoni, 1990) and induces mesoderm in area opaca explants (Stern et al., 1995). In addition, local application of activin initiates the formation of an ectopic primitive streak (Ziv et al., 1992; Cooke et al., 1994). However, as in *Xenopus*, activin in chick has not been shown to have the spatial or temporal distribution expected of an endogenous inducer (Mitrani and Shimoni, 1990; Connolly et al., 1995).

Recently, the chick homolog of *Vg1* (chick *Vg1* or *cVg1*) was identified (Shah and Dodd, 1995; Seleiro et al., 1996) and proposed to be involved in primitive streak formation. COS cells expressing *cVg1* were shown to stimulate initiation of a primitive streak when grafted to an ectopic site in the chick blastoderm (Seleiro et al., 1996). However, the early distribution of *cVg1* in the blastoderm appears inconsistent with a role in primitive streak formation (Seleiro et al., 1996). Furthermore, it remains unclear whether cVg1 can evoke a change in cell fate or whether it causes formation of an ectopic primitive

streak by recruitment of cells that were predestined to contribute to the normal primitive streak.

Here, we show that *cVg1* is expressed in the PMZ of the prestreak blastoderm, as expected of a molecule with a role in primitive streak formation. Using a heterologous expression system, we show that cVg1 protein can initiate the formation of a primitive streak which gives rise to a complete second embryonic axis. Moreover, we have used cell marking experiments and in vitro assays to examine the cellular mechanism by which cVg1 directs axial development. Cells that give rise to the ectopic primitive streak change their pattern of gene expression and undergo novel, centripetally directed cell movements. Purified cVg1 protein induces the expression of mesodermal markers in epiblast explants in vitro. Our results support the proposal that endogenous cVg1 plays a role in the formation of the primitive streak and show that cVg1 can act by inducing cells to adopt a primitive streak fate. Furthermore, our experiments allow us to separate experimentally two apparently independent effects of cVg1: its ability to initiate centripetal cell movements and the expression of organizer markers. Finally, the results of applying ectopic cVg1 do not mimic completely the results of PMZ transplantations and reveal cellular events in primitive streak development that require other signals.

MATERIALS AND METHODS

Embryos

Fertile White Leghorn chicken eggs (SPAFAS, CT) were incubated at 38°C. Prestreak embryos [stage X-XIV] were staged according to Eyal-Giladi and Kochav (1976) and older embryos [stage 2-20] according to Hamburger and Hamilton (1951).

Xenopus embryos were generated by standard procedures (Wu and Gerhart, 1991) and cultured to appropriate stages according to Nieuwkoop and Faber (1967).

Molecular cloning of *cVg1*

Degenerate primers were designed for conserved amino acid stretches within the mature domains of *Xenopus Vg1* and other closely related BMPs.

Vg-5′: WQDWIIA:
    5′-CGAATTCTGGCA(G/A)GA(T/C)TGGAT(T/C/A)AT(T/C/A)GC-3′
Vg-3′: HAIVQT:
    5′-CGGATCCGT(T/C)TGNA(T/C)(T/G/A)ATNGC(C/A)TG-3′

1 µg of plasmid DNA from an amplified stage 4 Hensen's node library (Hume and Dodd, 1993) was used as template in a PCR reaction ((1) 3 cycles: 95°C, 1 minute; 37°C, 1 minute; 72°C, 2 minutes; (2) 32 cycles: 95°C, 40 seconds; 50°C, 1 minute; 72°C, 1 minute; (3) 72°C, 10 minutes).

The amplified product was used as a probe to screen 500,000 colonies of the stage 4 Hensen's node library and an embryonic day 2 notochord library (C. R. H. and J. D., unpublished). cDNAs were sequenced by the Sanger dideoxy method (Sequenase kit, USB) and analyzed using Geneworks software (Intelligenetics). One cDNA with significant homology to *Xenopus Vg1* was studied further and subsequently named *chick Vg1* (*cVg1*). The largest *cVg1* clone isolated was 1.4 kb in length and included the entire coding sequence. The nucleotide sequence of *cVg1* has the GenBank accession number U55871.

One cDNA, clone 2.1.8, containing an open reading frame predicting a 373 amino acid product was analyzed further. The predicted protein product is 54% identical to *Xenopus* Vg1 overall, with 74%
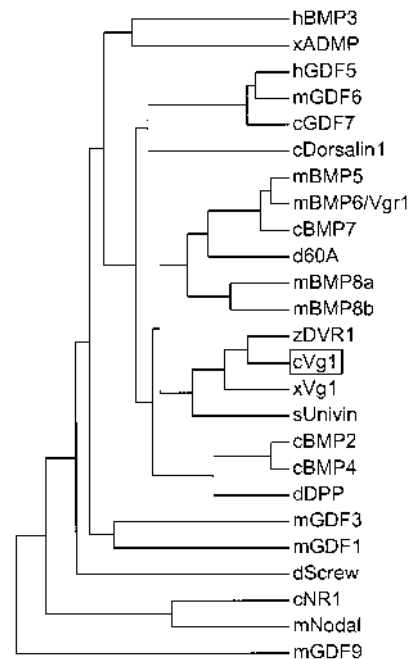

Fig. 1. Dendrogram of BMP family members. Mature domain sequences were compared starting at the first conserved cysteine residue. References for sequences can be found in Hogan (1996). Sequences were analyzed using Geneworks software.

identity in the predicted mature domain. This domain also shares 83% amino acid identity with zebrafish Vg1 (zDVR-1; Helde and Grunwald, 1993) (Fig. 1). Other closely related BMP family members share less amino acid identity in the mature domain with clone 2.1.8: sea urchin univin (66%), chick BMP2 (60%), chick BMP4 (59%), mouse BMP6 (59%), and chick BMP7 (58%) (Fig. 1). In vitro transcription and translation of *cVg1* cDNA with rabbit reticulocyte lysate generated a protein product of 42 kDa, the size expected from the open reading frame of the sequence (data not shown). These results suggest that clone 2.1.8 represents the *chick Vg1* (*cVg1*) gene. The independent cloning of *cVg1* has been reported (Seleiro et al., 1996, GenBank accession #U73003).

Whole-mount in situ hybridization

In situs on chick embryos using digoxigenin-labelled riboprobes were performed as previously described (Harland, 1991; Hume and Dodd, 1993; Thery et al., 1995). Whole-mount embryos were then processed for wax or frozen sectioning at 10 µm and 15 µm, respectively.

DNA constructs

A 30 base pair sequence coding for the myc-epitope (EQKLISEEDL) (Evans et al., 1985) was introduced four amino acids downstream of the putative cleavage site of the *cVg1* cDNA by PCR to facilitate protein detection with the anti-myc MAb, 9E10 (Evans et al., 1985). Internal primers were designed, each containing half of the myc sequence and *cVg1* sequence from the epitope insertion site.

Vg-myc-A: 5′-GCGATATCAGCTTCTGCTCGTTGTAAGCACTCC-TCCTCCTCCT-3′
Vg-myc-B: 5′-CGGATATCCGAGGAGGACCTGGTCCCCGTCAC-ACCGAGCAACC-3′

The N-terminal fragment generated with the T7 vector primer and Vg-mycA and the C-terminal fragment generated with Vg-mycB and the SP6 vector primer were joined by their primer-derived *Eco*RV sites (underlined) and ligated together to form *cVg1-myc* which was then subcloned into the pMT23 expression vector (Hume and Dodd,

1993). The integrity of the resulting construct was verified by sequencing.

A chimeric construct containing the N-terminal fragment of myc-tagged dorsalin-1 (dsl[myc]; Basler et al., 1993) and the C-terminal fragment of *cVg1-myc* was generated by ligation of fragments to form *dsl-cVg1*. This construct contains the cleavage site and four subsequent amino acids of dorsalin-1, the myc-epitope and the remainder of the mature domain encoded by cVg1.

COS cell production of cVg1 protein

COS-1 cells were transfected using Lipofectamine reagent (GIBCO). 2×10^6 cells on 15 cm dishes were transfected with 20 µg plasmid DNA for 5 hours followed by addition of medium containing 20% calf serum and incubation overnight. 10-15 ml serum-free medium per dish were conditioned for 2 days then cleared of cellular debris by centrifugation at 2500 *g* and stored at 4°C.

COS-1 cell-conditioned media were affinity purified on 9E10 (anti-myc)-Affigel (BioRad) columns and the eluate was desalted and concentrated. Purified protein concentration was below the level detectable by a modified Bradford assay (BioRad) but each batch was active and all experiments described here were performed with a single batch, permitting the use of dilution equivalents. Purified cVg1 was analyzed by western blot probed with the anti-*Xenopus* Vg1 mAb FC.14F6 or with an anti-*Xenopus* Vg1 polyclonal antibody (Tannahill and Melton, 1989), revealing bands at approximately 18 kDa and 50 kDa (not shown), indicating the cleaved product and the unprocessed precursor, respectively. The two antibodies produced identical results.

Western blots

Samples were prepared from COS cell supernatant, affinity-purified protein, chick embryo lysates, and *Xenopus* oocyte supernatants and cell lysates. To make lysates, oocytes (10 per sample) or embryos (pools of 100 embryos from stages XII and XIII) were homogenized in 0.1M TRIS buffer containing EDTA (1mM) and PMSF (1mM). Samples (aliquots of: 1-6 µl COS cell supernatant; 0.1-6 µl purified cVg1; 10 embryo equivalents embryo lysate; 5 µl oocyte supernatant and 0.5 oocyte equivalents of oocyte lysate) were boiled in sample buffer and separated by 15% SDS-PAGE. The gels were electroblotted onto Hybond-ECL nitrocellulose (Amersham). Blots were probed with anti-Vg1 monoclonal antibody (FC.14F6) diluted 1:2000 in Blotto and developed by chemiluminescence (Amersham).

Xenopus animal cap assay

Stage 8-9 blastula caps were treated with conditioned media or purified protein diluted with 0.5× MMR for 4-6 hours at room temperature. Explants were then transferred to 0.1× MMR and cultured for up to 2 days with frequent changes of medium.

For histological analysis, some explants were fixed with 4% paraformaldehyde and stained in whole-mount with 12/101 antibody (Kintner and Brockes, 1984) diluted 1:1000. Signal was detected by HRP-linked secondary antibodies and DAB substrate reaction.

Molecular analysis was performed as described (Wilson and Melton, 1994) using RT-PCR on RNA isolated from treated caps. *Brachyury* is a general mesodermal marker (Smith et al., 1991). *Muscle actin* is a marker of somitic muscle (Stutz and Sphor, 1986). *Goosecoid* (Blumberg et al., 1991), *chordin* (Sasai et al., 1994) and *noggin* (Smith and Harland, 1992) are markers of dorsal mesoderm. *EF1α* was used as a loading control (Krieg et al., 1989). Primers for *chordin* were: upstream-ACAGCATAGGCAGCTGTG and downstream-GTGTGCTTGGACAAGAGG (25 cycles). PCR products were resolved on 5% non-denaturing acrylamide gels and were detected by autoradiography.

Chick axis initiation assay

2×10^5 COS-1 cells were plated on 35 mm dishes 24 hours prior to transfection. Cells were transfected with 1 µg plasmid DNA using Lipofectamine reagent (GIBCO). After 24 hours, transfected cells were trypsinized and counted. Groups of 500 cells were allowed to aggregate overnight in hanging 20 µl drops of medium. These small aggregates were rinsed in serum-free medium and grafted, one per blastoderm, to the marginal zone of embryos (stage X-stage 4) at 180° from the prospective posterior pole, which was marked with carbon particles. Embryos were grown in modified New culture (New, 1955; Stern and Ireland, 1981) and assayed by morphology and in situ hybridization using *cVg1*, *cWnt8C* (Hume and Dodd, 1993), *brachyury* (Kispert et al., 1995), *goosecoid* (Izpisua-Belmonte et al., 1993) and *HNF3β* (Ruiz i Altaba et al., 1995) riboprobes.

Chick mesoderm induction assay

This assay was performed essentially as described (Stern et al., 1995). Epiblast explants were taken from stage XII lateral area opaca. The explants were cultured in rat tail collagen and medium 199 (GIBCO) in the presence of purified cVg1 or activin for 40 hours at 37°C, 5% CO_2. Explants were then processed for whole-mount in situ hybridization (Thery et al., 1995) using a full-length chick *chordin* riboprobe (K. J. L. and T. Jessell, unpublished data). Other markers of axial mesoderm also labelled hypoblast in this assay and could therefore not be used as diagnostic probes.

Fate mapping experiments

A quantitative staging system (Hatada and Stern, 1994) was used to locate the prospective notochord/head process or surface ectoderm/extraembryonic tissue cell populations to be labelled. Notochord/head process precursor cells are found at the midline. At stage X and XI, they are found near the posterior end of the area pellucida. By stage XII this population has moved to about 50% of the distance between the anterior border of the hypoblast and the posterior end of the embryo. The surface ectoderm/extraembryonic tissue cell population can be labelled at the midline about 90 distance units from the posterior end of the embryo, regardless of the embryonic stage.

The carbocyanine dyes 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indo-carbocyanine perchlorate (DiI, Molecular Probes) and 3,3′-dioctadecyl oxacarbocyanine perchlorate (DiO-C18, Molecular Probes) were used as described previously (Hatada and Stern, 1994). Label was always applied from the dorsal side of the embryo and DiI and DiO were used interchangeably in the two cell populations in different experiments. Some embryos were processed histologically to confirm the localization of the labelled cells after photo-oxidation of 3,3′-diaminobenzidine (DAB) as described previously (Stern, 1990).

Xenopus oocyte production of cVg1 protein

cDNAs were subcloned into pSP64TEN and linearized to produce transcription templates (Krieg and Melton, 1984). Synthetic RNAs were produced using Megascript (Ambion). Oocytes were prepared and injected with RNA as described (Kessler and Melton, 1995). Medium was collected from injected oocytes after 48 hours in culture and medium and cell lysates were subjected to western blot analysis.

RESULTS

Distribution of *cVg1* during embryogenesis

To determine whether *cVg1* is expressed at stages when primitive streak formation is initiated, we examined expression in chick embryos from prestreak to late embryo stages.

Expression of *cVg1* before and during primitive streak formation

The earliest expression of *cVg1* was detected before the formation of the primitive streak. At stage X, the earliest stage examined, *cVg1* is expressed at very low level in the posterior
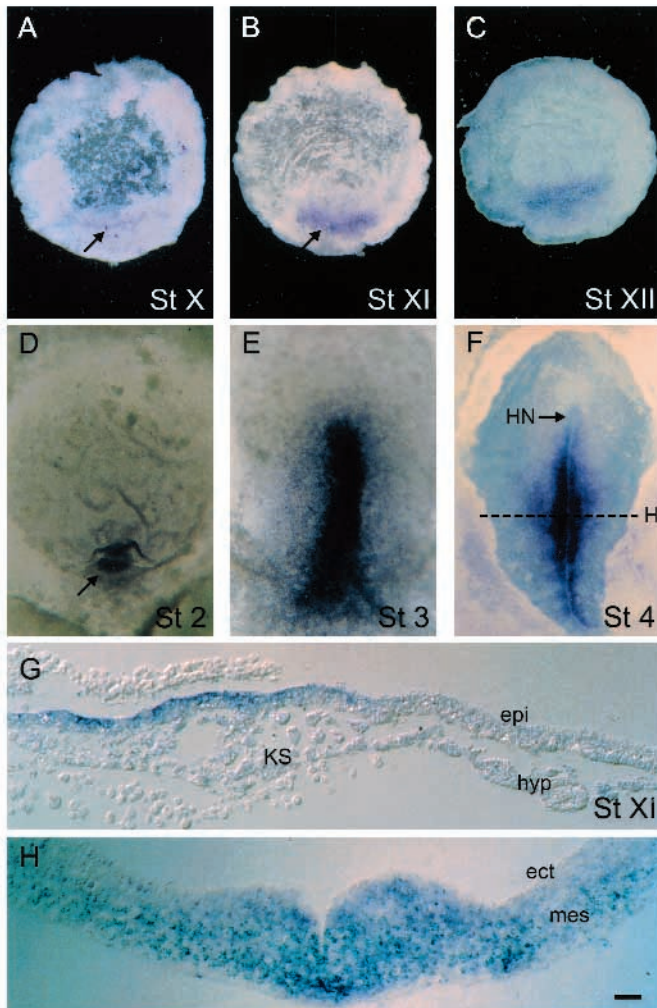
Fig. 2. Distribution of cVg1 in the chick gastrula. (A) Ventral view of a stage X embryo shows low level expression of *cVg1* (arrow) localized in the posterior of the embryo and slightly obscured by the overlying yolky germ wall. (B) At stage XI, the localized expression of *cVg1* in the PMZ is apparent (arrow). (C) By stage XII, the domain of expression of *cVg1* expands laterally and posteriorly. (D) At stage 2, transcript is localized to the primitive streak (arrow). (E) In a stage 3 embryo, *cVg1* is expressed in the primitive streak. (F) In late stage 4 embryos, *cVg1* is expressed in the primitive streak and in emerging lateral mesoderm (see also H) but begins to be downregulated in Hensen's node (arrow). (G) A sagittal section of a stage XI embryo shows *cVg1* localized to the epiblast of the posterior marginal zone. (H) Stage 4, transverse section through the primitive streak at site marked in F shows cVg1 expression in the forming lateral mesoderm. A-H are all in situ hybridizations using cVg1 antisense probe. Control embryos incubated without probe or with sense probe did not show labelling. Embryos incubated with other RNA probes did not display this pattern of expression. Abbreviations: HN, Hensen's Node; KS, Koller's Sickle; epi, epiblast; hyp, hypoblast; ect, ectoderm; mes, mesoderm. Scale bar: A-C, 300 μm; D,E, 120 μm; F, 150 μm; G,H, 15 μm.

blastoderm (Fig. 2A). During stages XI and XII, expression of *cVg1* increases and becomes clearly localized to the epiblast of the posterior marginal zone (Fig. 2B,C,G). During stages X to XIII the hypoblast extends anteriorly from the posterior margin. At stage XII, *cVg1* is found predominantly in the epiblast layer

of the PMZ (Fig. 2G). At stage 2, as the primitive streak begins to form, *cVg1* becomes concentrated in cells of the primitive streak (Fig. 2D). *cVg1* is expressed at high levels in cells throughout the primitive streak during its elongation and also in mesodermal cells lateral to the primitive streak (Fig. 2E,F,H). At stage 4⁻, *cVg1* is expressed in Hensen's node but, by stage 4, prior to regression of the node, *cVg1* becomes downregulated in the node and anterior primitive streak (Fig. 2F). Thus, *cVg1* is expressed before primitive streak formation, selectively in cells that are thought to induce or organize the primitive streak. In addition, *cVg1* is expressed in cells of the primitive streak itself, including progenitors of the future axial and paraxial mesoderm.

Expression of *cVg1* after primitive streak formation

Unlike its homologs in *Xenopus* and zebrafish, *cVg1* is dynamically expressed in the embryo during later development (Shah and Dodd, 1995; Seleiro et al., 1996). *cVg1* is strongly expressed throughout the unsegmented paraxial mesoderm (Fig. 3A,B) and is rapidly downregulated in the rostral halves of prospective somites just prior to segmentation (Fig. 3B). *cVg1* is maintained in the caudal region of epithelial somites for several hours and is then downregulated (Fig. 3B,C). At stage 17, *cVg1* is detected in the somitic myotome as it forms from the dermomyotome (not shown). At this stage, *cVg1* is also expressed in the mesenchyme of the branchial arches and in the myocardium of the heart (not shown). *cVg1* is transiently expressed in the notochord from stage 14 to stage 17 (Fig. 3D).
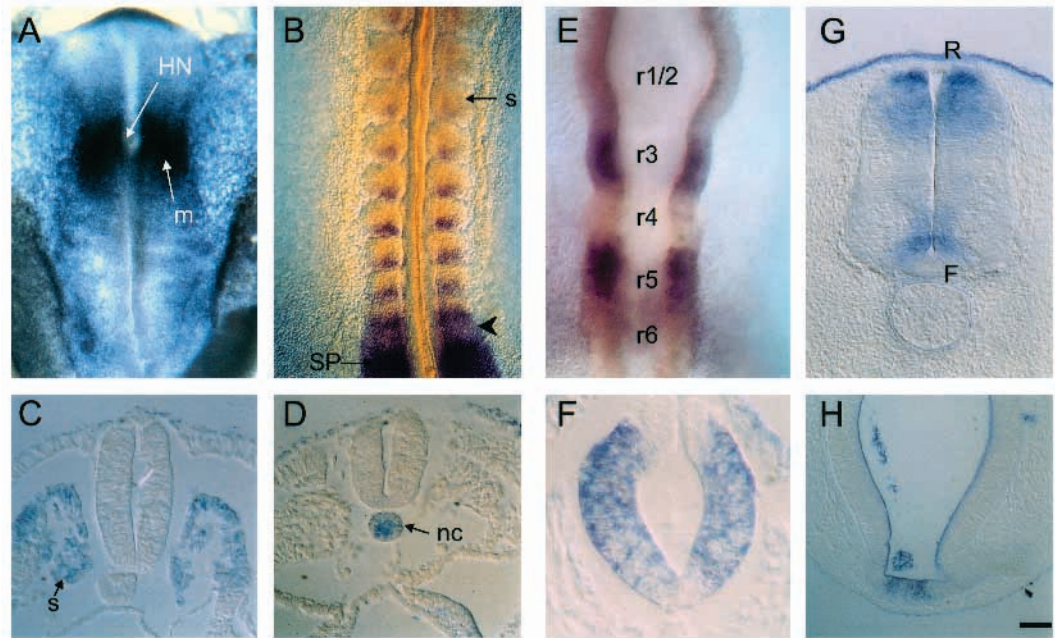
cVg1 also has a patterned distribution in the nervous system. A broad domain in the hindbrain at stage 8 (not shown) becomes restricted to rhombomeres (r) 3 and r5 by stage 10 (Fig. 3E,F) and, with the exception of the floor plate and dorsal midline, is found throughout the dorsoventral extent of the neural tube in r3 and r5 (Fig. 3F). At stage 15, expression in the hindbrain is downregulated (not shown) and, by stage 17, *cVg1* is found ventrally, lateral to the floor plate in the hindbrain and the spinal cord (Fig. 3G), and in the midline of the rostral diencephalon (Fig. 3H). At stage 18, expression is detected in the dorsal one-third of the spinal cord as well (Fig. 3G). In the periphery at stage 18, *cVg1* is expressed in coalescing sympathetic ganglia (not shown).

Synthesis of cVg1 by COS Cells

To generate recombinant cVg1 protein for use in biological assays, COS-1 cells were transiently transfected and conditioned media and transfected cells were harvested and assayed. Native *cVg1* tagged with the myc epitope did not direct the secretion of protein as detected by western blot (Fig. 4A). To enhance the secretion of cVg1 protein, we generated a chimeric cDNA containing the pro-region and cleavage site of chick *dorsalin-1* (Basler et al., 1993) and the potential ligand domain of *cVg1* (the chimeric construct, referred to as *dsl-cVg1*, was also myc tagged). Media conditioned by COS cells transfected with *dsl-cVg1* contained high levels of myc-tagged protein, assessed by western blot (Fig. 4A). Thus, in contrast to previously published findings (Saliero et al., 1996), it was possible to generate a source of soluble cVg1 protein.
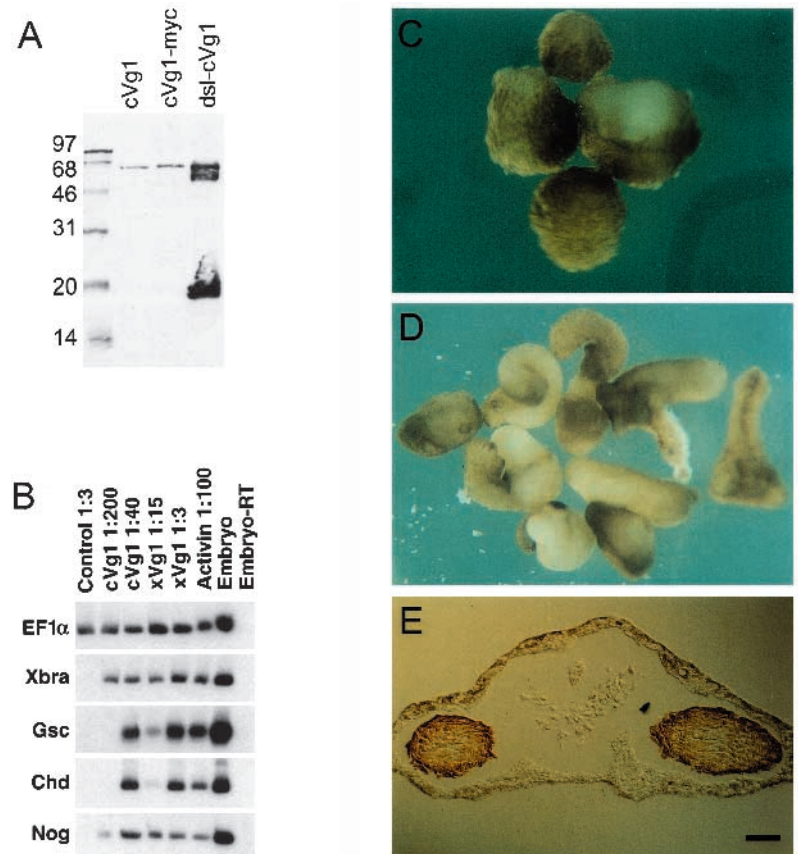
To determine whether COS-generated cVg1 protein is biologically active, we initially examined whether, like mature *Xenopus* Vg1, cVg1 produced from dsl-cVg1 can induce mesoderm in animal cap explants of *Xenopus* blastulae. Treated animal caps were assayed for mesoderm induction by

Fig. 3. Distribution of *cVg1* in the developing chick embryo. (A) At stage 6, *cVg1* transcript is found in forming paraxial mesoderm. (B) A stage 10 embryo shows the expression of *cVg1* in the segmental plate (SP) and forming somite. *cVg1* is rapidly downregulated in the rostral region of the newly forming somite (arrowhead) and cannot be detected in rostral halves of somites after segmentation (s). *cVg1* is more gradually lost from caudal halves of somites.
(C) Transverse section through the caudal region of a stage 14 embryo shows the expression of *cVg1* throughout the immature epithelial somite (s).
(D) Further rostral in the same stage 14 embryo, *cVg1* is absent from somites but is transiently expressed in the notochord (nc).
(E) By stage 10, *cVg1* expression becomes restricted to rhombomeres (r) 3 and 5 in the hindbrain. (F) A transverse section through r3 of the embryo shown in A shows expression of *cVg1* throughout the dorsoventral axis with the exception of the midline. (G) At stage 17-18 *cVg1* is expressed in the ventral spinal cord, adjacent to the floor plate (F) and in dorsal regions adjacent and ventral to roof plate (R). (H) *cVg1* is expressed in the ventral midline of the rostral diencephalon at stage 17. Scale bar: A, 300 μm; B, 125 μm; C,D, F-H, 30 μm; E, 60 μm.

Fig. 4. Mesoderm-inducing activity of COS cell-generated cVg1 protein. (A) Western blot of COS cell-conditioned media probed with anti-Vg1 mAb, FC1.4F6. Lane 1 is a marker ladder. Conditioned media from COS cells transfected with native *cVg1, cVg1-myc* and *dsl-cVg1* were applied to the gel. (B) Induction of early mesoderm markers in *Xenopus* animal cap explants assessed by RT-PCR. 1:200 of affinity-purified cVg1 protein induced expression of *brachyury (Xbra)* and *noggin (Nog)* in explants cultured to the late gastrula stage. A 5-fold higher concentration of cVg1 induced expression of the organizer markers *goosecoid (Gsc), chordin (Chd)* and *noggin* in addition to *brachyury*. This activity mimics the profile of *Xenopus* Vg1 supernatants obtained from oocytes injected with *activin-Xenopus Vg1*. Control supernatants did not induce mesoderm markers.
(C-E) Animal caps cultured to the tadpole stage.
(C) Untreated animal caps develop into ciliated epidermis.
(D) Caps cultured in the presence of *dsl-cVg1*-transfected COS cell-conditioned medium, diluted 1:10 undergo elongation movements indicative of dorsal mesoderm induction. (E) Section of cVg1-treated cap labelled with the muscle marker 12/101 indicating skeletal muscle.
Scale bar: C, 200 μm; D, 250 μm; E, 30 μm.

morphology, RT-PCR and immunocytochemistry. Caps treated with cVg1- or activinβA-conditioned medium underwent elongation movements suggestive of the convergence and

extension movements of induced axial mesoderm (Fig. 4D and not shown). The induction of early mesoderm markers was assessed by RT-PCR (Fig. 4B). At 1:200 of the stock solution

(see Methods), purified cVg1 induced the general mesoderm marker *brachyury* (Smith et al., 1991) and weakly induced *noggin*, which is normally expressed in the organizer (Smith and Harland, 1992). At a five-fold higher concentration, cVg1 induced *brachyury* expression in addition to the organizer markers *goosecoid* (Blumberg et al., 1991), *chordin* (Sasai et al., 1994) and *noggin*. Similar results were obtained with *Xenopus* Vg1.

Examination of cVg1-treated *Xenopus* caps cultured to a stage equivalent to the tadpole stage revealed the presence of notochord cells (not shown) and skeletal muscle labelled with mAb 12/101 (Kintner and Brockes, 1984) (Fig. 4E). Control explants did not undergo elongation movements or express mesodermal markers (Fig 4B,C). These results demonstrate that cVg1 protein is a potent inducer of dorsal mesoderm in *Xenopus* ectoderm.

cVg1 directs primitive streak formation

The early expression of *cVg1* in the posterior marginal zone and the ability of cVg1 protein to induce dorsal mesoderm in the *Xenopus* animal cap assay suggested that cVg1 may represent an endogenous signal for axis formation in the chick.

Misexpression of *cVg1* in the marginal zone initiates ectopic primitive streak formation

To examine the role of cVg1 in the chick embryo, we tested the ability of an ectopic source of cVg1 protein to initiate and sustain primitive streak formation in whole blastoderms in culture. Hanging drop aggregates of COS cells, transiently transfected with control or *dsl-cVg1* constructs, were grafted to the area opaca or the marginal zone, at 180° from the putative posterior pole, which was marked with carbon for future identification or the middle of the area pellucida of stage X to stage 4 chick blastoderms. Embryos were assessed, at times from 6-48 hours, for the presence of an ectopic primitive streak by gross morphology and by in situ hybridization of genes normally expressed in the primitive streak.

In embryos in which COS cells transfected with *dsl-cVg1* were placed in the marginal zone, ectopic primitive streaks developed (56% of embryos, stages X to XIII, *n*=157; Table 1). Of these, 84% had two primitive streaks (Fig. 5A; Table 1) and 16% developed only a single primitive streak arising from the site of grafting (Table 1). Stage 2-4 embryos grafted with cVg1-expressing cells in the marginal zone developed normally without an ectopic or displaced primitive streak (Table 1). In contrast, grafts of COS cells transfected with *dsl-cVg1* but placed outside the marginal zone in the area opaca or in the middle of the area pellucida, did not initiate formation of an ectopic primitive streak (Fig. 5E,F).

Grafts of COS cells transfected with native *cVg1* or *dorsalin-1* did not perturb normal primitive streak development (not shown). Similarly, embryos at all stages grafted with control COS cells transfected with vector alone (*n*=50) produced a single primitive streak originating within 30° of the predicted posterior pole (Table 1).

cVg1-initiated primitive streak gives rise to a normal, mature axis

To assess whether ectopic primitive streaks resulting from misexpression of cVg1 in the marginal zone were normal, experimental embryos were probed with primitive streak markers.

Table 1. Ectopic cVg1 activity stimulates ectopic primitive streak formation in chick blastoderms

	Stage of host embryo (n)	% embryos showing ectopic axis development	% embryos with 2° axis also have 1° axis	% embryos with ectopic axis only
cVg1-expressing	X (18)	77	79	21
COS cells at	XI (30)	47	86	14
180° from	XII (47)	34	88	12
posterior pole	XIII (62)	56	94	6
	2-4 (22)	4 (n=1)	100	0
	Total (179)			
Control COS cells at 180°	X-XIII (50)	0	-	-
cVg1-expressing COS cell at 90°	XI-XII (10)	50	100	0

Development of ectopic primitive streaks was delayed relative to the primary primitive streak but appeared normal. At the morphological equivalent of stage 4 for the ectopic primitive streak, the expression patterns of *Cwnt-8C* (Hume and Dodd, 1993), *brachyury* (Kispert et al., 1995), *HNF3β* (Ruiz i Altaba et al., 1995) *goosecoid* (Izpisua-Belmonte et al., 1993) and *cVg1* (Seleiro et al., 1996; this paper) were similar to normal stage 4 embryos. *Cwnt-8C* was detected in the posterior two-thirds and *brachyury* and *cVg1* were expressed along the entire length of the ectopic primitive streak (Fig. 5D). As in the primary primitive streak, *cVg1* expression was then down-regulated in the tip of the ectopic primitive streak (Hensen's node) and both organizer markers, *goosecoid* (Fig. 5B) and *HNF3β* (not shown), were found in Hensen's node.

The use of small aggregates of COS cells to provide an ectopic source of cVg1 did not result in disruption of the embryo and it was possible to culture experimental embryos over extended periods. To determine whether ectopic primitive streaks initiated by cVg1 subsequently have the ability to form complete embryonic axes, 19 embryos were cultured for 30 hours or longer. In 11 of these, both the primary and secondary axes developed. Each axis formed a neural tube, a notochord and somites, assessed in whole mount and in sections (Fig. 5C and not shown) and expressed *brachyury* (Fig. 5D) and HNF3β (not shown) in the notochord. Thus, ectopically applied cVg1 initiates formation of a primitive streak that subsequently develops normally, giving rise to a full embryonic axis, sustainable as late as stage 9, the oldest stage examined.

cVg1 induces mesoderm in chick explants

The activity of cVg1-expressing COS cells in the primitive streak assay may result from the actions of cVg1 on chick blastoderm cells. Alternatively, the activity might reflect signalling by a COS cell-derived factor, induced in COS cells by cVg1 expression, or the co-ordinated activity of cVg1 and a COS cell-derived factor. The availability of purified cVg1 protein permitted us to determine whether cVg1 acts in the absence of COS cell signalling and directly on blastoderm cells. Purified recombinant cVg1 protein was tested for its ability to induce the expression of mesoderm markers in isolated chick extraembryonic epiblast (Stern et al., 1995). Explants of lateral area opaca epiblast, which normally give rise to extraembryonic tissue, were assayed for the expression of *chordin*, a specific marker for axial mesoderm. *Chordin* is normally expressed in Hensen's node and

one of its derivatives, the notochord (Fig. 6A,B) and is expressed in explants of Hensen's node in tissue culture (not shown; *n*=4/9). Control epiblast explants did not express *chordin* when cultured alone (Fig. 6C; *n*=0/12). However, when treated with affinity-purified cVg1 protein (1:25), explants expressed *chordin* (Fig. 6D; *n*=7/16), indicating that cells with axial mesodermal properties were induced. This result suggests that cVg1 acts directly on blastoderm cells to induce mesoderm.

Development of ectopic primitive streak by cVg1 involves a change in cell fate

In normal development, the cellular interactions leading to primitive streak formation are unclear. Potential mechanisms include initiation of the primitive streak by an inductive interaction between the PMZ and epiblast cells and/or by recruitment of predetermined cells to the site of primitive streak inititiation. The results described above indicate that ectopic cVg1 can evoke the formation of an ectopic primitive streak with normal properties and that cVg1 can induce a fate change in epiblast cells. To determine whether cells that give rise to the cVg1-evoked ectopic primitive streak are subverted from a different cell fate or are recruited from amongst the cells that would normally contribute to the primary primitive streak, we labelled discrete populations of blastoderm cells with carbocyanine dyes and examined their fates in the presence and absence of an ectopic source of cVg1 (Fig. 7A-C).

Cells adjacent to the ectopic source of cVg1 change their fate

Cells at the anterior pole of the blastoderm give rise to surface ectoderm and extraembryonic tissues and normally maintain that position or migrate laterally as development proceeds (Hatada and Stern, 1994). Anterior pole cells were labelled with DiO or DiI and the COS cell graft was immediately placed just anterior to the label. In control embryos, in which COS cells were transfected with vector alone, and in embryos without transplants, labelled cells remained near the graft or migrated a short distance laterally (Fig. 7B). In contrast, labelled cells adjacent to cVg1-secreting grafts followed migratory paths away from the graft toward the center of the blastoderm and contributed to the ectopic primitive streak and axial mesoderm (Fig. 7A,C,E,G,H). This result indicates that cVg1 induced local cells to undergo a change in migratory behavior and cell fate such that they contribute only to the secondary primitive streak and its derivatives.

To determine whether recruitment of prespecified axial cells plays a role in the development of the ectopic axis, notochord precursors in the midline of the posterior half of the embryo (Hatada and Stern, 1994) were labelled with DiI or DiO (Fig. 7A). These cells contributed to the primary primitive streak and embryonic axis only. They did not migrate to the ectopic primitive streak (Fig. 7D,F,H). Although it is not possible to label all the prospective axial cells of the primary axis, this result suggests that recruitment of prespecified axial cells is not responsible for ectopic axis formation induced by cVg1.

Active cVg1 protein is synthesized by *Xenopus* oocytes

Taken together, the results described above provide evidence that cVg1 could act as an endogenous inducer of primitive streak. However, native cVg1 is not efficiently processed or

secreted by COS-1 cells. In *Xenopus*, Vg1 has the activities and distribution required for an endogenous dorsal mesoderm inducer, but mature protein is not detected in the embryo. To determine whether functional cVg1 protein is produced and secreted in the chick embryo, lysates of whole embryos (stages X-XII) were analyzed by western blot probed with the MAb FC1.4F6. This antibody recognizes an epitope in the mature domain of *Xenopus* Vg1 (Tannahill and Melton, 1989) and also binds zebrafish Vg1 zDVR-1 (Dohrmann et al., 1996). Unprocessed cVg1 precursor protein was detected in blastoderm lysates, but cleaved protein was not detected (not shown; see also Seleiro et al., 1996).

Since the level of mature protein present in lysates of pools of embryos is below the sensitivity of the anti-Vg1 antibody, we wished to determine whether native *cVg1* encodes a functional protein product that can be processed and secreted. The *Xenopus* oocyte system provides a potentially more abundant source of protein and has been used previously to express Vg1 homologues (Kesseler and Melton, 1995; Dohrmann et al., 1996). Native *cVg1* was therefore expressed in *Xenopus* oocytes and the oocyte supernatant examined by western blotting for evidence of mature protein. Soluble *Xenopus* Vg1 protein obtained by expression of a chimeric transcript in oocytes is a potent inducer of dorsal mesoderm in vitro (Kessler and Melton, 1995). We therefore examined the medium conditioned by native cVg1 for mesoderm-inducing activity in the *Xenopus* animal cap assay to test the activity of mature cVg1 obtained by cleavage of native cVg1 precursor.

Xenopus oocytes expressing native *cVg1* and medium conditioned by the oocytes were probed on western blots with anti-Vg1 IgG. Supernatant from oocytes injected with native cVg1 contained bands at approximately 18 kDa and 50 kDa, indicating cleaved and uncleaved proteins, respectively (Fig. 8A). Thus, native cVg1 contains the sequences necessary for precursor cleavage and secretion in *Xenopus* oocytes.

To test whether mature protein generated from native cVg1 is active, we examined supernatant from oocytes injected with *cVg1* for mesoderm-induction activity in *Xenopus* animal caps. We compared cVg1 activity to that of supernatants from oocytes secreting *Xenopus* Vg1 and activin. Supernatants collected from oocytes expressing native cVg1 or dsl-cVg1 induced *muscle actin*, a marker of dorsal mesoderm (Fig. 8B). The levels of inducing activity were similar to those in supernatants from oocytes injected with *activin* or with *activin-Xenopus Vg1* chimeric constructs. Native *Xenopus Vg1* did not produce detectable mature protein.

These results indicate that the *cVg1* gene encodes a functional ligand with activity similar to that of the related TGFβ family members, *Xenopus* Vg1 and activin.

DISCUSSION

We have described the distribution of cVg1 in the developing chick embryo and begun to examine its potential functions. Prior to primitive streak formation, cVg1 is expressed in one region of the epiblast, the PMZ, that initiates primitive streak formation when transplanted to an ectopic site in the blastoderm and is thought to contain the signals responsible for primitive streak initiation (Spratt and Haas, 1960; Azar and Eyal-Giladi, 1979; Eyal-Giladi and Khaner, 1989; Khaner and
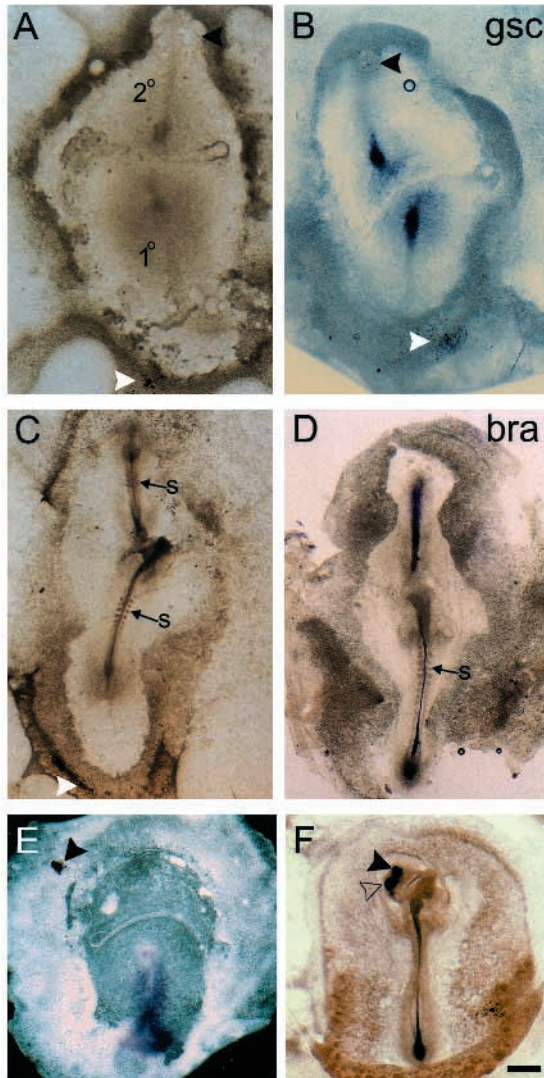
Fig. 5. cVg1 initiates primitive streak formation in cultured chick embyros. Hanging drop aggregates of *dsl-cVg1*-transfected COS cells were grafted to the anterior margin of prestreak embryos. (A) After 24 hours, a second primitive streak has formed, originating near the graft site. (B) In situ hybridization of another embryo 24 hours after implant showing *goosecoid (gsc)* gene expression in both organizers. (C) Cultured for 48 hours, cVg1-initiated streaks give rise to embryonic axial structures, including neural tube, notochord and somites (s). (D) A double embryo labelled with *brachyury (bra)* riboprobe. Both notochords express *bra*. (E) Stage 4 embryo in which COS cells expressing cVg1 were placed in the area opaca (black arrowhead). Only the primary axis developed. (F) Embryo cultured for 35 hours after COS cells expressing cVg1 were placed in the middle of the area pellucida (black arrowhead). The embryos in both E and F were labelled with the *bra* probe. In these examples trapping of probe by the COS cell aggregate was observed. This occurred sporadically in the experiments and was sometimes useful for COS cell localization. When cVg1 was expressed in the area pellucida, *bra* was induced in adjacent ectoderm (open arrowhead in F). Black arrowheads indicate the site of COS cell graft. (Circle near black arrowhead in B is an air bubble introduced during mounting.) White arrowheads indicate carbon, marking predicted posterior pole of embryo. Scale bar: A,B,E, 250 µm; C,D, 450 µm; F, 350 µm.

zDVR-1 suggests that they are homologs. Like *Xenopus* Vg1 and zDVR-1, cVg1 is a potent inducer of dorsal mesoderm in *Xenopus* animal caps. In addition, cVg1 is capable of initiating normal axial development when ectopically expressed in the anterior marginal zone of chick blastoderms. This evidence and the early expression of *cVg1* support the conclusion that *cVg1* is a true homolog of *Xenopus Vg1*. Unlike its homologs in *Xenopus* and zebrafish, *cVg1* is dynamically expressed in the embryo after gastrulation. Zygotic transcription of *Xenopus Vg1* (Rebagliati et al., 1985; Tannahill and Melton, 1989) or *zDVR-1* (Helde and Grunwald, 1993) has not been observed. Thus, in chick, Vg1 appears to have been adopted for later roles in development.

Is cVg1 secreted in the chick embryo?

Although cVg1 precursor protein has been detected in early

Eyal-Giladi, 1989). Small aggregates of cells transfected with *cVg1* and grafted into a host blastoderm induce cell types that display gene expression patterns and cell movements typical of normal primitive streak cells, leading to the development of a complete ectopic primitive streak that generates a well-developed secondary embryo. Fate mapping experiments demonstrate that cells undergo a change in fate to contribute to the ectopic primitive streak. We also show that purified cVg1 protein induces node and notochord gene marker expression in extraembryonic epiblast explants. Together, these results suggest that cVg1 may initiate primitive streak formation by a direct inductive mechanism.

Is cVg1 the homologue of *Xenopus* Vg1?

The sequence similarity among *cVg1, Xenopus Vg1* and

Fig. 6. Purified cVg1 protein induces the organizer marker chordin in vitro. (A) At stage 4, *chordin* transcript is expressed in Hensen's node. (B) A transverse section of a stage 10 embryo shows strong expression of *chordin* in the notochord. (C) Untreated lateral area opaca epiblast does not express *chordin* after culture for 40 hours. (D) Area opaca explants treated with purified cVg1, diluted 1:25, express *chordin*. Scale bar: A, 150 µm; B-D, 30 µm.
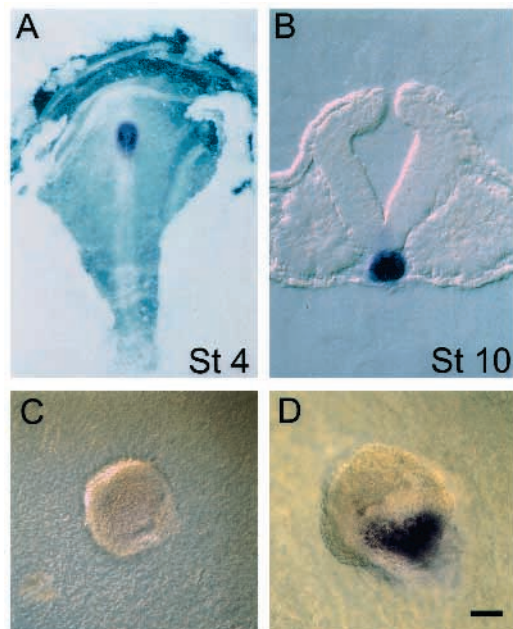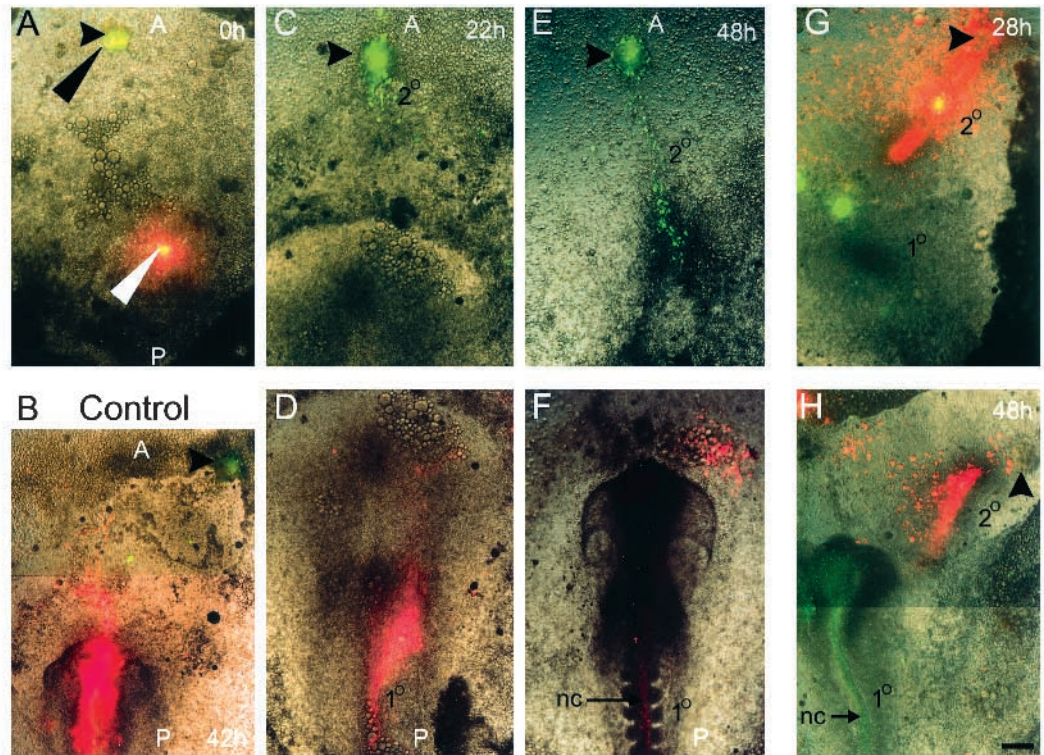
Fig. 7. Fate mapping during cVg1-initiated primitive streak formation. (A) Labelled embryo photographed immediately after placement of graft and used here to show experimental procedure. Carbocyanine dye (DiI or DiO) was injected into the blastoderm to label notochord precursors (long white arrowhead, DiI in this example). A second dye (DiO or DiI) was used to label area pellucida cells in the anterior blastoderm (long black arrowhead, DiO in this example). The graft (indicated by black arrowhead) was positioned just peripheral to the dye, immediately after labelling. (B,C) Control embryo in which mock-transfected COS cells were implanted, after 42 hours. Notochord developed (red) but anterior cells (green) have not moved (compare with panels E and H). (A,C-F) Time course of a single embryo labelled with DiI (red) posteriorly and DiO (green) anteriorly and visualized with epifluorescence (0 hour point shown in A). (C) Embryo shown in A at 22 hours. Anterior region of embryo shows anterior cells (2°) begin movements toward the center of the blastoderm. (D) Posterior region of embryo shown in (C). Labelled cells in the epiblast contribute to the primary primitive streak (1°). (E) Anterior region of same embryo at 48 hours. Labelled cells near the graft have continued centripetal movements. (F) Posterior region of same embryo at 48 hours. Labelled cells contribute to the primary (1°) notochord (nc). Labelled cells anterior to the head are likely to be cells of the hypoblast labelled initially and migrating anteriorly to form extraembryonic structures. (G) Another embryo, shown at 28 hours after having been labelled anteriorly with DiI (red) and posteriorly with DiO (green). Only anterior labelled cells give rise to the ectopic primitive streak. (H) Another embryo, shown 48 hours after having been labelled anteriorly with DiI (red) and DiO (green) posteriorly. Posterior cells contribute only to the primary notochord, whereas anterior cells give rise to the ectopic primitive streak. Anterior (A) and posterior (P) poles of the embryos are marked in each set of panels. Black arrowheads mark the position of COS cell aggregates. Scale bar: A,C-F, 250 µm; B,G,H, 350 µm.

chick embryos (Seleiro et al, 1996; this study, data not shown), mature cVg1 protein has not been demonstrated in extracts of whole embryos. Similarly, mature *Xenopus* Vg1 and zDVR-1 protein cannot be detected in *Xenopus* and zebrafish and may reflect the sensitivity of the anti-Vg1 antibodies and the low concentrations of mature protein likely to be present in the embryo. *Xenopus Vg1* and *zDVR-1* are widely expressed in *Xenopus* and zebrafish embryos (Tannahill and Melton, 1989; Helde and Grunwald, 1993) and, in *Xenopus*, post-translational modification of a widely distributed precursor protein is thought to account for localized activity of Vg1 (Dale et al., 1993; Thomsen and Melton, 1993). However, it is unclear whether such a mechanism is required in chick, given the patterned expression of *cVg1*.

It remains unclear whether cVg1 is processed in the chick embryo. However, the finding that cVg1 is processed by *Xenopus* oocytes injected with RNA indicates that cVg1 contains a functional cleavage site. Native zDVR-1 is also processed and secreted by *Xenopus* oocytes (Dohrmann et al., 1996). cVg1 cleavage in oocytes appears at least as efficient as that of zDVR-1 and of activin-*Xenopus* Vg1 chimeras all of which are more efficient than that of *Xenopus* Vg1. These results may reflect species differences in sequences that regulate Vg1 processing. The sequence of both the cleavage

site and the neighboring pro-region has been shown to regulate processing of *Xenopus* Vg1 and zDVR-1 (Dohrmann et al., 1996). Although both proteins have cleavage sites that fit the consensus protease recognition motif (R-X-K/R-R; Vey et al., 1992), there are additional sequences immediately upstream of the cleavage site which appear to interfere with efficient processing. zDVR-1 contains two overlapping motifs, whereas the cleavage site of *Xenopus* Vg1 is preceded by two potential pseudo protease recognition motifs (Dohrmann et al., 1996). In contrast, the cVg1 cleavage site (RRRR) fits the consensus and is without additional upstream sequences resembling those that are thought to interfere with processing in *Xenopus* and zebrafish. The differences in putative regulatory sites suggest that cleavage of cVg1 may be more efficient than that of either *Xenopus* Vg1 or zDVR-1.

Ectopic cVg1 activity may mimic a downstream signal from the PMZ

Only one primitive streak forms in a normal embryo despite the early competence of the entire periphery to respond to an axis-inducing signal. This may be due to the localized expression of an inducing signal but is also thought to reflect the spreading activity of a factor that inhibits primitive streak formation in cells adjacent to the developing primitive streak
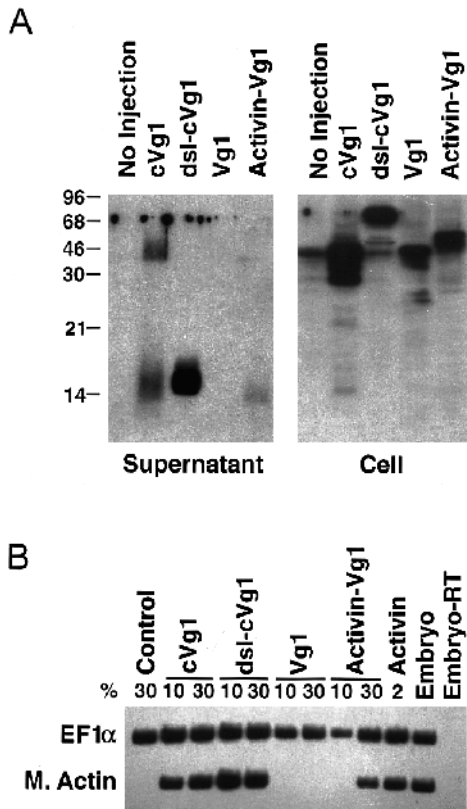
A

96 —
68 —
46 —
30 —
21 —
14 —

Supernatant   **Cell**

B

Fig. 8. cVg1 is processed and secreted when expressed in *Xenopus* oocytes. (A) Western blot analysis of conditioned supernatants and cell extracts probed with anti-*Xenopus* Vg1 MAb, FC.14F6. Native *Xenopus* Vg1 (Vg1) is not cleaved or secreted into the medium. An activin-*Xenopus* Vg1 chimera (Activin-Vg1) is cleaved and secreted. cVg1 precursor is abundant within the cell and both precursor and cleaved mature domain are secreted into the supernatant. Expression of *dorsalin-cVg1* (dsl-cVg1) chimera leads to enhanced secretion of the mature peptide. Multiple bands ranging from 50 to 70 k Da in the cell pellets lanes are thought to represent unprocessed precursor Vg1 protein carrying different length propeptides and undergoing different amounts of breakdown. (B) Induction of muscle actin in animal caps by oocyte-conditioned supernatants diluted to 10% and 30%. Supernatant conditioned by oocytes expressing native *Xenopus* Vg1 does not induce muscle actin (M.Actin), but medium conditioned by chimeric activin-*Xenopus* Vg1 and diluted to 30% does induce expression of muscle actin. CVg1 produced from either native *cVg1* or *dsl-cVg1* induces muscle actin when supplied as a 10% dilution of supernatant. A 2% dilution of supernatant containing activin induces muscle whereas control supernatant has no effect. EF1α was used as a control for loading and reverse transcription. Embryo and embryo-RT are additional positive and negative controls, respectively.

(Khaner and Eyal-Giladi, 1986; Ziv et al., 1992). Thus, transplantation of a active PMZ into stage X or stage XI embryos, to sites 90° or more from the PMZ, results in double embryo formation. Transplantation at less than 90° results in failure of one axis (Khaner and Eyal-Giladi, 1989). By stage XII, the ability of the lateral marginal zone to produce an ectopic primitive streak in response to a active PMZ graft is lost (Khaner and Eyal-Giladi, 1986). It is not possible to distinguish whether loss of response in the stage XII embryo reflects a spread of the putative inhibitory signal or, through a different

mechanism, a loss of competence by the blastoderm. The molecular bases for the apparent inhibition and loss of competence have not been determined. Both could be established by the primitive streak-initiating signal itself, by a signal from cells induced to form primitive streak or by a distinct signal from the PMZ. The inhibitory signal may act to prevent adjacent tissue from responding to the primitive streak-inducing signal or by preventing production of primitive streak-inducing activity in adjacent tissue.

In our experiments, induction of an ectopic primitive streak by cVg1 at 180° to the PMZ was usually accompanied by normal development of the primary primitive streak (see Table 1). Furthermore, when discrete grafts of small pellets of COS cells secreting cVg1 were placed at 90°, or less, to the posterior pole of stage XI and stage XII embryos and induced ectopic axis formation, both the primary and the secondary primitive streaks always formed (see Table 1). This result contrasts with the finding that, when larger COS cell pellets were implanted, even at 180°, in stage XI and XII chicks, 47% of experimental animals had ectopic axes but, of these, only 3% (13% of total) had double axes, retaining the primary axis (Saleiro et al., 1996). However, large pellets of COS cells may preclude local restriction of the ectopic signal, increasing the potential for inhibition of more than one primitive streak or may cause cellular disruption so that only a single primitive streak forms.

Our finding that the secondary primitive streaks induced by cVg1 can form in close proximity to the primary primitive streak suggests that cVg1 is not itself responsible for the inhibition of primitive streak formation that accompanies experimental grafting of a PMZ within 90° of the posterior pole. Moreover, this result indicates that the blastoderm cells adjacent to the primary primitive streak are competent to generate a secondary primitive streak. Thus, the inhibitory activity of the PMZ may act by preventing signalling by adjacent marginal zone. The ability of ectopic cVg1 to circumvent inhibition may result from the fact that cVg1 mimics a signal of the PMZ.

The idea that cVg1 mimics a PMZ graft signal or a downstream effector of other PMZ signals is supported by the finding that embryos are able to respond to COS cell-generated cVg1 at later stages (XII and XIII) than to PMZ grafts. It is possible that cVg1 overcomes loss of competence/inhibition in stage XII and XIII embryos because transgene expression by transfected COS cells is high, whereas tissue transplants may express endogenous genes at much lower levels and/or in a regulated manner. However, the extended competence of the embryo to respond to ectopic cVg1 may reflect the time normally required to produce cVg1 by the PMZ graft.

Cellular mechanism of primitive streak initiation

The results of our fate mapping experiments during the initiation of primitive streak development by ectopic cVg1, combined with the activity of cVg1 protein on blastoderm explants, indicate that ectopic primitive streak initiation occurs through an inductive mechanism in which the fate of cells in the anterior region of the embryo is subverted to a primitive streak fate. In *Xenopus*, Vg1 activity suggests that it acts as a Nieuwkoop center signal which induces the organizer (Thomsen and Melton, 1993; Kessler and Melton, 1995). The implication of a TGFβ receptor (TARAM-A) in zebrafish organizer formation also supports a role for TGFβ signalling

during axial development (Renucci et al., 1996). Several genes have been identified that are potentially downstream of *Xenopus* Vg1 in organizer induction. One such gene is *chordin*, which has been shown to induce axial development in *Xenopus* (Sasai et al., 1994). In chick epiblast explants as well as in *Xenopus* animal caps, cVg1 induces organizer markers including *chordin*. The activation by cVg1 of *chordin* and other organizer and mesoderm markers is consistent with the proposed role of Vg1 in *Xenopus* and may thus lead to the formation and organization of the primitive streak, and within it Hensen's node, the chick organizer (see Hara, 1978).

Multiple steps involved in primitive streak formation and the roles of cVg1

In the normal embryo, primitive streak formation is preceded (between stages XI and XIII) by convergence of epiblast cells to the posterior midline, followed by movement along the midline towards the centre of the blastoderm (Hatada and Stern, 1994). Most of the cells that will contribute to the organizer, Hensen's node (prospective prechordal mesendoderm, notochord, some somite cells), undergo these movements and reach the center of the blastoderm before the primitive streak starts to form. A second population of cells that contributes to the organizer constitutes a sparse middle layer beneath the posterior epiblast at stages XII-XIII and does not begin its anterior movement until the primitive streak starts to form at stage 2 (Izpisúa-Belmonte et al., 1993). This latter population contributes mostly to endoderm. Within the stage 3^+-4 Hensen's node, most cells express the organizer markers, *chordin*, *HNF-3β* and *goosecoid*, suggesting that expression of these markers is acquired by cells irrespective of their origin and of the route by which they reach the node.

Our dye-labelling experiments show that ectopic expression of cVg1 in the embryo causes host cells adjacent to the cVg1-expressing COS cell graft to migrate centripetally. Moreover, we have observed that the COS cell pellet itself is sometimes carried by these movements towards the center of the embryo. These movements can occur even in those cases when an ectopic primitive streak does not appear to form (e.g. Fig. 7C-F).

At the same time, it appears that neither the formation of a visible primitive streak nor the co-ordinated movements of epiblast cells are essential prerequisites for expression of organizer markers. When treated with either cVg1 (this study) or activin (Stern et al., 1995), isolated explants of extraembryonic epiblast can acquire expression of *chordin* or *goosecoid*. Moreover, when pellets of COS cells expressing cVg1 are grafted either into the area opaca or in the central area of the area pellucida, away from the marginal zone, they do not initiate formation of a primitive streak even though, in the area pellucida, the *brachyury (bra)* riboprobe was induced (Fig. 5F), and in vitro experiments reveal that the extraembryonic area opaca epiblast can respond to cVg1 by expressing *chordin*. These observations suggest that induction of organizer markers may not be sufficient for the initiation of a primitive streak and that the marginal zone may contain additional factors or constraints that are also important. Thus, there is a property, or properties, of the marginal zone essential for primitive streak initiation, as first suggested by Spratt and Haas (1960). In conclusion, cVg1, by its localization and its ability to initiate primitive streak formation by inducing organizer markers and

appropriate cell movements, represents a candidate signalling molecule of the PMZ. However, other features of the PMZ, essential to positioning of the primitive streak, and found in the PMZ, are not mimicked by cVg1 and illustrate the complexity of this multistep process.

REFERENCES

Azar, Y. and Eyal-Giladi, H. (1979). Marginal zone cells-the primitive streak-inducing component of the primary hypoblast in the chick. *J. Embryol. Exp. Morph.* **52**, 79-88.

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

Blumberg, B., Wright, C. V., De Robertis, E. M. and Cho, K. W. (1991). Organizer-specific homeobox genes in Xenopus laevis embryos. *Science* **253**, 194-6.

Connolly, D. J., Patel, K., Seleiro, E. A., Wilkinson, D. G. and Cooke, J. (1995). Cloning, sequencing, and expressional analysis of the chick homologue of follistatin. *Dev. Genetics* **17**, 65-77.

Cooke, J., Takada, S. and McMahon, A. (1994). Experimental control of axial pattern in the chick blastoderm by local expression of Wnt and activin: the role of HNK-1 positive cells. *Dev. Biol.* **164**, 513-27.

Dale, L., Matthews, G. and Colman, A. (1993). Secretion and mesoderm-inducing activity of the TGF-beta-related domain of Xenopus Vg1. *EMBO J.* **12**, 4471-80.

Dohrmann, C. E., Hemmati-Brivanlou, A., Thomsen, G. H., Fields, A., Woolf, T. M. and Melton, D. A. (1993). Expression of activin mRNA during early development in Xenopus laevis. *Dev. Biol.* **157**, 474-83.

Dohrmann, C. E., Kessler, D. S. and Melton, D. A. (1996). Induction of axial mesoderm by zDVR-1, the zebrafish orthologue of Xenopus Vg1. *Dev. Biol.* **175**, 108-117.

Ecochard, V., Cayrol, C., Foulquier, F., Zaraisky, A. and Duprat, A. M. (1995). A novel TGF-beta-like gene, fugacin, specifically expressed in the Spemann organizer of Xenopus. *Dev. Biol.* **172**, 699-703.

Evans, G. I., Lewis, G. K., Ramsay, G. and Bishop, J. M. (1985). Isolation of monoclonal antibodies specific for human c-myc protooncogene product. *Mol. Cell Biol.* **5**, 3610-3616.

Eyal-Giladi, H. and Khaner, O. (1989). The chick's marginal zone and primitive streak formation. II. Quantification of the marginal zone's potencies – temporal and spatial aspects. *Dev. Biol.* **134**, 215-21.

Eyal-Giladi, H. and Kochav, S. (1976). From cleavage to primitive streak formation: a complementary normal table and a new look at the first stages of the development of the chick. I. General morphology. *Dev. Biol.* **49**, 321-37.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol* **88**, 49-92.

Hara, K. (1978) Spemann's organizer in birds. In *Organizer – a Milestone of a Half-Century from Spemann. Ed O. Nakamura & S Toivonen.* pp221-265. Amsterdam: North Holland.

Harland, R. (1991). In situ hybridization: an improved whole-mount method for Xenopus embryos. *Methods in Cell Biol.* **36**, 675-685.

Hatada, Y. and Stern, C. D. (1994). A fate map of the epiblast of the early chick embryo. *Development* **120**, 2879-89.

Helde, K. A. and Grunwald, D. J. (1993). The DVR-1 (Vg1) transcript of zebrafish is maternally supplied and distributed throughout the embryo. *Dev. Biol.* **159**, 418-26.

Hogan, B. L. M. (1996). Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev.* **10**, 1580-1594.

Hume, C. R. and Dodd, J. (1993). *Cwnt-8C*: a novel Wnt gene with a potential

role in primitive streak formation and hindbrain organization. *Development* **119**, 1147-60.

Izpisua-Belmonte, J. C., De Robertis, E. M., Storey, K. G. and Stern, C. D. (1993). The homeobox gene goosecoid and the origin of organizer cells in the early chick blastoderm. *Cell* **74**, 645-59.

Jones, C. M., Kuehn, M. R., Hogan, B. L., Smith, J. C. and Wright, C. V. (1995). Nodal-related signals induce axial mesoderm and dorsalize mesoderm during gastrulation. *Development* **121**, 3651-3662.

Kessler, D. S. and Melton, D. A. (1995). Induction of dorsal mesoderm by soluble, mature Vg1 protein. *Development* **121**, 2155-64.

Khaner, O. and Eyal-Giladi, H. (1986). The embryo-forming potency of the posterior marginal zone in stages X through XII of the chick. *Dev. Biol.* **115**, 275-81.

Khaner, O. and Eyal-Giladi, H. (1989). The chick's marginal zone and primitive streak formation. I. Coordinative effect of induction and inhibition. *Dev. Biol.* **134**, 206-14.

Kintner, C. R. and Brockes, J. P. (1984). Monoclonal antibodies identify blastemal cells derived from dedifferentiating muscle in newt limb regeneration. *Nature* **308**, 67-69.

Kispert, A., Ortner, H., Cooke, J. and Herrmann, B. G. (1995). The chick brachyury gene: developmental expression pattern and response to axial induction by localized activin. *Dev. Biol.* **168**, 406-15.

Krieg, P., Varmum, S., Wormington, M. and Melton, D. A. (1989). The mRNA encoding elongation factor 1α (EF1α) is a major transcript at the mid-blastula transition in Xenopus. *Dev. Biol.* **133**, 93-100.

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

Mitrani, E. and Shimoni, Y. (1990). Induction by soluble factors of organized axial structures in chick epiblasts. *Science* **247**, 1092-1094.

New, D. A. T. (1955). A new technique for the cultivation of the chick embryo in vitro. *J. Embryol. Exp. Morph.* **3**, 326-331.

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

Rebagliati, M. R., Weeks, D. L., Harvey, R. P. and Melton, D. A. (1985). Identification and cloning of localized maternal RNAs from Xenopus eggs. *Cell* **42**, 769-77.

Renucci, A., Lemarchandel, V. and Rosa, F. (1996). An activated form of typeI serine/thronine kinase receptor TARAM-A reveals a specific pathway involved in fish head organiser formation. *Development* **122** 3735-3743.

Ruiz i Altaba, A., Placzek, M., Baldassare, M., Dodd, J. and Jessell, T. M. (1995). Early stages of notochord and floor plate development in the chick embryo defined by normal and induced expression of HNF-3 beta. *Dev. Biol.* **170**, 299-313.

Sasai, Y., Lu, B., Steinbeisser, H., Geissert, D., Gont, L. K. and De Robertis, E. M. (1994). Xenopus chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* **79**, 779-90.

Seleiro, E. A. P., Connolly, D. J. and Cooke, J. (1996). Early developmental expression and experimental axis determination by the chicken Vg1 gene. *Current Biology* **6**, 1476-1486.

Shah, S. B. and Dodd, J. (1995). Distribution and characterization of chick Vg1. *Dev. Biol.* **170**, 772.

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

Smith, J. C., Price, B. M., Van, N. K. and Huylebroeck, D. (1990). Identification of a potent Xenopus mesoderm-inducing factor as a homologue of activin A. *Nature* **345**, 729-31.

Smith, W. C., McKendry, R., Ribisi, S. Jr and Harland, E. M. (1995). A nodal-related gene defines a physical and functional domain within the Spemann organizer. *Cell* **82**, 37-46.

Smith, W. C. and Harland, R. M. (1992). Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in Xenopus embryos. *Cell* **70**, 829-40.

Spratt, N. T. and Haas, H. (1960). Integrative mechanisms in development of early chick blastoderm. I. Regulative potentialities of separated parts. *J. Exp. Zool.* **145** 97-137

Spratt, N. T. and Haas, H. (1965). Germ layer formation and the role of the primitive streak in the chick. I. Basic architecture and morphogenetic tissue movements. *J. Exp. Zool.* **158**, 9-38.

Stern, C. D. (1990). The marginal zone and its contribution to the hypoblast and primitive streak of the chick embryo. *Development* **109**, 667-682.

Stern, C. D. and Ireland, G. W. (1981). An integrated experimental study of endoderm formation in avian embryos. *Anat. Embryol.* **163**, 245-63.

Stern, C. D., Yu, R. T., Kakizuka, A., Kintner, C. R., Mathews, L. S., Vale, W. W., Evans, R. M. and Umesono, K. (1995). Activin and its receptors during gastrulation and the later phases of mesoderm development in the chick embryo. *Dev. Biol.* **172**, 192-205.

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

Tannahill, D. and Melton, D. A. (1989). Localized synthesis of the Vg1 protein during early Xenopus development. *Development* **106**, 775-85.

Thery, C., Sharpe, M. J., Batley, S. J., Stern, C. D. and Gherardi, E. (1995). Expression of HGF/SF, HGF1/MSP, and c-met suggests new functions during early chick development. *Dev. Genetics* **17**, 90-101.

Thomsen, G. H. and Melton, D. A. (1993). Processed Vg1 protein is an axial mesoderm inducer in Xenopus. *Cell* **74**, 433-41.

Vakaet, L. (1970). Cinephotomicrographic investigations of gastrulation in the chick blastoderm. *Archives de Biologie* **81**, 387-426.

Vakaet, L. (1984) The initiation of gastrular ingression in the chick blastoderm. *Amer. Zool.* **24** 555-562.

Vey, M., Orlich, M., Adler, S., Klenk, H-D., Rott, R. and Garten, W. (1992). Hemagglutinin activation of pathogenic avian influenze viruses of serotype H7 requires the protease recognition motif R-X-K/R-R. *Virology* **188**, 408-13.

Weeks, D. L. and Melton, D. A. (1987). A maternal mRNA localized to the vegetal hemisphere in Xenopus eggs codes for a growth factor related to TGF-beta. *Cell* **51**, 861-7.

Wilson, P. A. and Melton, D. A. (1994). Mesodermal patterning by an inducer gradient depends on secondary cell-cell communication. *Curr. Biol.* **4**, 676-686.

Wu, M. and Gerhart, J. (1991). Raising *Xenopus* in the laboratory. *Meth. Cell Biol.* **36**, 3-17.

Ziv, T., Shimoni, Y. and Mitrani, E. (1992). Activin can generate ectopic axial structures in chick blastoderm explants. *Development* **115**, 689-94.