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Localized synthesis of the Vg1 protein during early *Xenopus* development

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Summary

The *Xenopus* Vg1 gene encodes a maternal mRNA that is localized to the vegetal hemisphere of both oocytes and embryos and encodes a protein related to the TGF-β family of small secreted growth factors. We have raised antibodies to recombinant Vg1 protein and used them to show that Vg1 protein is first detected in stage IV oocytes and reaches maximal levels in stage VI oocytes and eggs. During embryogenesis, Vg1 protein is synthesized until the gastrula stage. The embryonically synthesized Vg1 protein is present only in vegetal cells of an early blastula. We find that Vg1 protein is glycosylated and associated with membranes in the early embryo. Our results also suggest that a small proportion of the full-length Vg1 protein is cleaved to give a small peptide of $M_r = \sim 17 \times 10^3$. These results support the proposal that the Vg1 protein is an endogenous growth-factor-like molecule involved in mesoderm induction within the amphibian embryo.

Key words: Vg1, transforming growth factor beta, localization, mesoderm induction, *Xenopus*.

Introduction

Formation of mesoderm in the marginal zone of the early amphibian embryo is thought to involve inductive interactions whereby mesoderm is induced in the animal hemisphere by signals emanating from the vegetal hemisphere (Dale & Slack, 1987; Gurdon *et al.* 1985; Nakamura *et al.* 1971; Nieuwkoop, 1969; Smith *et al.* 1985). Regional specification of the mesoderm into dorsal mesoderm, characterized by notochord and muscle and into ventral mesoderm characterized, by kidney and blood, is thought to arise from differences in the signals produced by dorsal and ventral vegetal blastomeres, respectively (Boterenbrood & Nieuwkoop, 1973; Dale & Slack, 1987; Dale *et al.* 1985). The signals that mediate mesoderm induction have been shown to be diffusible factors in trans-filter experiments (Grunz & Tacke, 1986; Gurdon, 1989; Slack *et al.* 1987). Recently, it has been determined that soluble growth factors can induce mesoderm in isolated animal pole explants (Godsave *et al.* 1988; Grunz *et al.* 1988; Kimelman & Kirschner, 1987; Rosa *et al.* 1988; Slack *et al.* 1987; Smith, 1987).

Basic fibroblast growth factor (bFGF), from mammalian sources, has previously been shown to induce mainly ventral mesoderm in animal pole explants (Grunz *et al.* 1988; Kimelman & Kirschner, 1987; Slack *et al.* 1987). More recently, *Xenopus* bFGF has also been shown to have comparable effects (Kimelman *et al.* 1988; Slack & Isaacs, 1989). More dorsal mesoderm, such as notochord, is rarely found in animal pole explants treated with bFGF, and so it is probable that another molecule, alone or together with bFGF, is responsible for inducing dorsal mesoderm. A clue to potential nature of the dorsal inducing signal was given by the observation that human transforming growth factor-β1 (TGF-β1) can act synergistically with bFGF to give increased levels of muscle in animal pole explants (Kimelman & Kirschner, 1987). Furthermore, a closely related molecule, TGF-β2 (but not TGF-β1), can alone induce mesoderm as judged by muscle actin expression (Rosa *et al.* 1988). Thus a combination of bFGF and TGF-β may be sufficient to induce both dorsal and ventral mesoderm.

A number of TGF-β-like molecules have been identified in *Xenopus*. XTC-MIF (Xenopus tissue culture-mesoderm-inducing factor) is a factor isolated from a *Xenopus* tissue culture cell line that induces large amounts of dorsal mesoderm (Smith, 1987; Smith *et al.* 1988). Interestingly, antibodies to TGF-β2 but not TGF-β1 can reduce the efficacy of XTC-MIF action suggesting that XTC-MIF is a TGF-β2-like molecule (Rosa *et al.* 1988). A novel TGF-β-related mRNA, called TGF-β5, has recently been identified in *Xenopus* embryos (Kondaiah *et al.* 1989). TGF-β5 is highly expressed at the onset of neurulation and is abundantly expressed by the cell line that secretes XTC-MIF. In a screen for mRNAs that have spatially restricted distributions within the egg, another mRNA related to TGF-β was isolated (Rebagliati *et al.* 1985; Weeks & Melton, 1987). This mRNA, called Vg1, becomes localized during oogenesis to form a tight subcortical shell in the vegetal hemisphere of stage VI oocytes (Melton, 1987*a*). Upon egg maturation and subsequent fertilization, this tight shell of Vg1 mRNA is released such that all the vegetal blastomeres in the cleaving egg inherit

Vg1 mRNA (Rebagliati *et al.* 1985; Weeks & Melton, 1987).

The greatest similarity between Vg1 and the TGF-β family lies in the last 120 carboxyl terminal amino acids, and includes 7 conserved cysteines which are presumably used for dimer formation. A pair of basic amino acids which are recognized as a cleavage site for the release of a small secreted peptide in the TGF-βs are also conserved in the putative Vg1 protein (see Massagué, 1987; Rizzino, 1988; Sporn *et al.* 1987 for reviews of TGF-β structure and function). The putative Vg1 protein has both a *N*-terminal hydrophobic signal sequence for insertion into the endoplasmic reticulum and three putative *N*-linked glycosylation sites suggesting that it may be secreted.

The role of the Vg1 protein in early development is unknown, but two observations suggest Vg1 is a candidate for a natural mesoderm inducer. First, the putative Vg1 protein bears great similarity to molecules that are known to be active in mesoderm-inducing assays, and secondly, Vg1 mRNA is localized within the cells of the early embryo that emits inductive signals. Therefore, we have proposed that the Vg1 protein may be secreted by vegetal cells to induce animal cells to form mesoderm (Weeks & Melton, 1987). In this paper, we show that Vg1 is a glycoprotein synthesized both in oocytes and in the vegetal blastomeres of early embryos. We also show that a small Vg1 peptide ($M_r = \sim 17 \times 10^3$) can be cleaved from the full-length Vg1 protein ($M_r = \sim 43 \times 10^3$). Additionally, we show that the full-length Vg1 protein can be secreted *in vitro*. These results are consistent with the proposed function of the Vg1 protein as a mesoderm-inducing signal within the early amphibian embryo.

Materials and methods

Embryological and radiolabelling methods

Xenopus embryos were obtained as described in Krieg & Melton (1985). All embryonic stages were according to Nieuwkoop and Faber (1967). Embryos were dissected in 1× MMR (Newport & Kirschner, 1982) on top of a layer of 1% agarose using watchmakers forceps. To label proteins in developing embryos, [^{35}S]-Trans-label (ICN) was concentrated 5- to 10-fold before microinjection. Synthetic mRNAs were dissolved at a concentration of 200 ng μl^{-1} in sterile water before microinjection (20 nl per egg). Embryos injected with mRNA 30 min before the first cleavage furrow formed were labelled for protein by a second injection of concentrated Trans-label 20–30 min later. Oocytes were staged as previously described (Dumont, 1972). Oocyte proteins were labelled essentially as before (Harvey *et al.* 1986; Melton, 1987*b*) except that Trans-label was substituted for [^{35}S]-methionine. Oocytes were placed in 96-well microtitre plates coated with 1 mg ml^{-1} BSA to prevent the non-specific absorption of secreted proteins.

Transcription of synthetic Vg1 mRNA

A chimeric Vg1 gene, consisting of Vg1 cDNA sequences from the *Bam*HI site at position +81 to the *Bst*EII at position +1106 and genomic sequences from the *Pst*I site at position +6 to the *Bam*HI site at position +81 (see Weeks & Melton, 1987 for numbering), was cloned into the transcription vector pSP64T (Krieg & Melton, 1984). After linearization, at the appropriate restriction site, synthetic capped mRNAs were generated as in Krieg & Melton (1984). Enzymes and other reagents were supplied by New England Biolabs, Promega Biotec and Amersham International.

Protein methods

Non-yolk *Xenopus* proteins were isolated essentially as described previously (Lane *et al.* 1979; Melton, 1987*b*). SDS–polyacrylamide gel electrophoresis, fluorography and other general methods were carried out as described in Goding (1986) and Harlow & Lane (1988).

Production of Vg1 fusion protein

The *Alu*I to *Rsa*I fragment of the Vg1 coding region (Weeks & Melton, 1987) encompassing the region related to TGF-β was cloned into the T7 expression vector pET3a and transformed into the bacterial strain pLysS (Rosenberg *et al.* 1987). Cultures of transformed bacteria were grown and induced as suggested (Rosenberg *et al.* 1987). Bacteria were pelleted by centrifugation and boiled in SDS–sample buffer before loading on to a preparative SDS–polyacrylamide gel. After electrophoresis, proteins were visualized with cold 4 M-sodium acetate (Higgins & Dahmus, 1979) and the recombinant Vg1 protein band was excised. The acrylamide gel slice was macerated in 10 vol. of 50 mM-Tris pH 8·0, 150 mM-NaCl, 5 mM-DTT, 0·1 mM-EDTA, 0·1% SDS and protein eluted overnight at room temperature. Elutate was removed and proteins precipitated overnight at −20°C by addition of 5 vol. of cold acetone. Recombinant Vg1 protein was pelleted by centrifugation, dried and dissolved in PBS.

Antibody production

To generate rabbit polyclonal antibodies against Vg1 protein, 100 μg of the recombinant Vg1 protein, made as above, was mixed with an equal volume of complete Freund's adjuvant and injected directly into popliteal lymph nodes (Sigel *et al.* 1983). 10 and 14 weeks later the rabbits were boosted with a subcutaneous injection of recombinant Vg1 protein (100 μg) in Freund's incomplete adjuvant. Immune sera was collected 1 week after each boost. The IgG fraction was purified by ammonium sulfate precipitation and DEAE Affi-Gel blue (Bio-Rad) column chromatography as described by the manufacturer.

Monoclonal antibodies were prepared from RBF/DNJ mice (Jackson laboratory) previously immunized subcutaneously with the recombinant Vg1 protein (50 μg) in Freund's adjuvant. Immune spleen cells were fused with the FoxNY myeloma cell line (Taggart & Samloff, 1983) using standard polyethylene glycol-mediated fusion methods (Goding, 1986; Harlow & Lane, 1988), and hybridomas were selected for in Optimem media (Gibco) with adenine, aminopterin and thymidine (Taggart & Samloff, 1983). Positive hybrids were identified by ELISA assay (Engvall, 1980) using Immulon II assay plates (Dynatech Laboratories Inc.) coated with 25 ng per well of recombinant fusion protein. An anti-mouse IgG antibody conjugated with alkaline phosphatase (Cappell) was used as the secondary antibody and after washing three times with 150 mM-NaCl, 50 mM-Tris pH 7·6, positive wells were identified by development with 1 mg ml^{-1} para-nitrophenol phosphate in 1 mM-$ZnCl_2$, 1 mM-$MgCl_2$, 0·1 M-glycine pH 10·4. Positive cultures were cloned at least two times by limiting dilution. Peritoneal tumors were induced by injection of 10^6 hybridoma cells into BALB/c mice previously primed with pristane 1 week beforehand. Mice were also gamma irradiated 2 days before injection of the

hybridoma cells to encourage formation of the tumor. After 1–2 weeks ascites fluid was taken from the mice and purified by ammonium sulfate precipitation and passage over protein-A sepharose as described in Harlow & Lane (1988).

Western blotting

Protein samples were separated on 10 or 15% gels and transferred to nitrocellulose sheets (Towbin *et al.* 1979). Blots were reversibly stained with 3% Ponceau S (Sigma) in 0·5% TCA and the required regions of the blot cut out. Blots were blocked in 1% low fat milk powder in 150 mM-NaCl, 50-mM-Tris pH 7·6 for 2 h before incubation with primary antibody solution (10–50 $\mu g\,ml^{-1}$) in the same solution for 2 h. After washing in 150 mM-NaCl, 50 mM-Tris pH 7·6, antibody binding was visualized with Vector Laboratories alkaline phosphatase ABC kit using NBT/BCIP substrates (Promega Biotec).

Immunoprecipitation

Immunoprecipitation techniques were modified from Lee *et al.* (1984). Labelled protein samples were diluted to 1 ml with cold 1× IP buffer (150 mM-NaCl, 1% NP40, 0·5% sodium deoxycholate, 0·1% SDS, 50 mM-Tris, pH 7·6) and antibody (2 μl of polyclonal serum or 1–2 μg of monoclonal antibody) added and left overnight at 4°C. 25 μl of protein-A sepharose beads (which had been preabsorbed to an oocyte protein extract) were added and rocked gently for 1 h at 4°C. Beads were pelleted and then washed three times (5–10 min each wash) in 1× IP buffer. After removal of all the liquid, an equal volume of SDS–sample buffer was added to the beads and samples boiled for 5 min before loading directly on to protein gels. For analysis of glycosylation, beads were washed an additional three times with 150 mM-NaCl, 50 mM-Tris pH 7·6 before resuspending in an equal volume of 100 mM-sodium acetate pH 5·2, 40 mM-EDTA, 0·4% Triton X-100, 0·5 mM-PMSF. 5 mU of endoglycosidase H (Boehringer Mannheim) or 250 mU of endoglycosidase F (Boehringer Mannheim) were then added and incubated overnight at 37°C. Samples were boiled in SDS sample buffer before loading onto gels as described above.

In vitro *translation*

Synthetic mRNAs were translated in rabbit reticulocyte lysates as described by the manufacturer (Promega Biotec) except that Trans-label (ICN) was used as the radioactive label. *In vitro* processing was carried out with canine pancreatic microsomal membranes (Promega Biotec) as suggested by the manufacturer. Some samples were treated with 100 $\mu g\,ml^{-1}$ proteinase K (Boehringer Mannheim) on ice for 1 h in either the presence or absence 0·1% Triton X-100. Proteinase K was inactivated by the addition of PMSF to 1 mM. Samples were immunoprecipitated in 1× IP buffer as described above. Determination of the subcellular location of the translated products in the membranes was carried out essentially as described by Teixidó *et al.* (1987).

Results

Generation of specific antibodies against the Vg1 protein

The complete amino acid sequence of the predicted translation product of the vegetally localized Vg1 mRNA has been deduced from its DNA sequence (Weeks & Melton, 1987; A. Fields, H. O'Keefe and D. Tannahill unpublished results). These data showed that the Vg1 protein is related to the TGF-β family of secreted growth factors. From the predicted protein sequence, it was anticipated that Vg1 protein would be processed from a full-length form of $M_r = \sim 42{\cdot}1 \times 10^3$ to a $M_r = \sim 13{\cdot}1 \times 10^3$ secreted form by cleavage at a pair of basic amino acids. This data also suggested that the Vg1 protein could be glycosylated and could undergo dimer formation either with itself or with another, as yet uncharacterized, TGF-β family member (see Fig. 1 for predicted protein structure of Vg1). Given that the small secreted peptide of TGF-β is responsible for its biological effects in other systems (Massagué, 1987; Rizzino, 1988; Sporn *et al.* 1987) we decided this would be the most appropriate region of the Vg1 protein with which to raise antibodies. Therefore, both polyclonal and monoclonal antibodies were made against the last 120 amino acids of the Vg1 protein expressed in the pET bacterial expression system (Rosenberg *et al.* 1987).

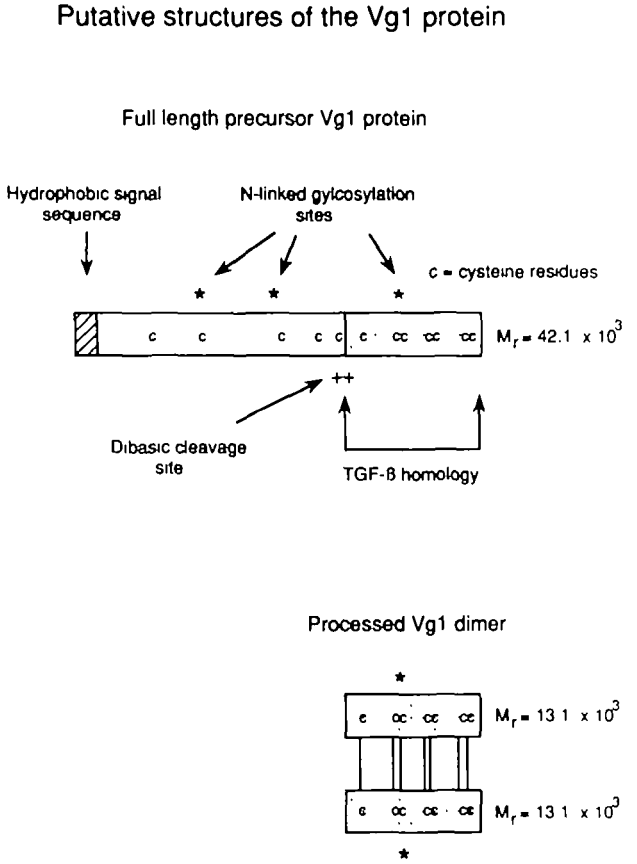

Fig. 1. The predicted structure of the Vg1 protein. The translation product of the Vg1 mRNA is predicted to be a long precursor ($M_r = \sim 42{\cdot}1 \times 10^3$) that is cleaved at two sites: (1) by removal of the hydrophobic signal sequence (shown cross-hatched) as the protein traverses into the endoplasmic reticulum and (2) by cleavage at a pair of basic amino acids (shown as ++) to release a small Vg1 monomer ($M_r = \sim 13{\cdot}1 \times 10^3$). The small Vg1 peptide is expected to form a dimer through any one of the 7 cysteines (marked c) as shown. Possible *N*-linked glycosylation sites are marked with a star. The region of similarity between Vg1 and TGF-β encompasses the region downstream of the basic cleavage site.

Western blotting experiments show that the resulting antisera specifically react only with bacterially expressed Vg1 protein and not with an unrelated protein (N-CAM) expressed in the same vector (Fig. 2A and B). Another band is often seen migrating at the predicted size for a dimer of bacterial Vg1 protein (Fig. 2B). A further proof of the specificity of the antisera is given by the observation that a protein of $M_r = \sim 35 \times 10^3$ can be immunoprecipitated from *in vitro* translation reactions directed with synthetic full-length Vg1 mRNA using polyclonal anti-Vg1 antisera but not with control preimmune antisera (Fig. 2C). The Vg1 protein made *in vitro* has a faster mobility in SDS–PAGE compared to the relative molecular mass predicted from the Vg1 amino acid sequence. This does not arise through premature termination of translation *in vitro* since the deglycosylated *in vivo* form of Vg1 protein also has this faster mobility (see Fig. 5). Four independent polyclonal antisera and three independent monoclonal antibodies against the Vg1 protein behave similarly in the above assays (data not shown).

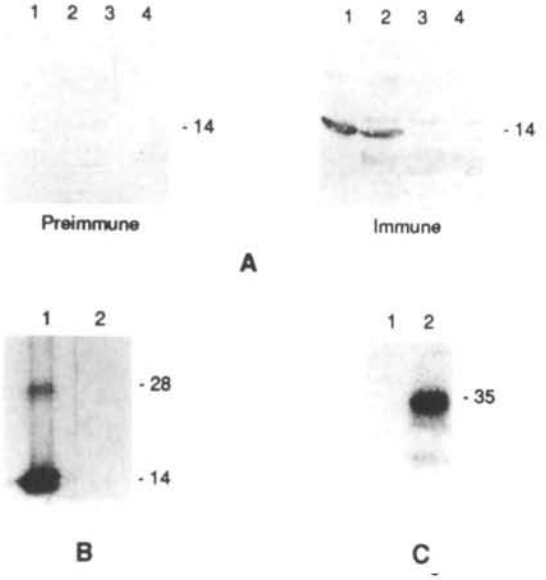

Fig. 2. Specificity of the anti-Vg1 antibodies. (A) Western blot probed with either preimmune rabbit sera or an anti-Vg1 polyclonal antisera. Lane 1 is the purified recombinant Vg1 antigen that was used for immunizations. Lane 2 is the *E. coli* cell strain expressing Vg1 protein. Lane 3 is the *E. coli* strain with vector alone not expressing any protein and lane 4 is the *E. coli* strain without vector. The anti-Vg1 antibody reacts with the recombinant Vg1 protein, $M_r = \sim 14 \times 10^3$, (lanes 1 and 2, immune blot). (B) Western blot of Vg1 (lane 1) or N-CAM (lane 2) protein expressed in *E. coli* and probed with an anti-Vg1 mouse monoclonal antibody. The antibody does not react with N-CAM protein expressed in the same vector, ruling out the possibility that it recognizes vector-derived sequences. (C) The rabbit anti-Vg1 antisera immunoprecipitates a protein of $M_r = \sim 35 \times 10^3$ from *in vitro* translations directed with synthetic Vg1 mRNA (lane 2), whereas control preimmune antisera does not (lane 1). Again the mouse monoclonal anti-Vg1 antibodies behave similarly (data not shown). In all these tests all 3 monoclonal antibodies and all 4 polyclonal antibodies behave similarly. Relative molecular mass ($\times 10^{-3}$) is indicated on the right-hand side.

Characterization of endogenous Vg1 protein

To determine whether the polyclonal and monoclonal antibodies recognize embryonically synthesized Vg1 protein, fertilized eggs were injected with [^{35}S]-Trans-label and allowed to develop until stage 8. Proteins labelled within this period were isolated and analyzed by immunoprecipitation techniques. Fig. 3A shows that a protein of $M_r = \sim 43 \times 10^3$ can be immunoprecipitated from labelled blastula extracts by both the monoclonal and polyclonal anti-Vg1 antibodies (lanes 1 and 2) but not with preimmune sera (lane 3). Fig. 3B shows that the protein immunoprecipitated by the anti-Vg1 antibody can be competed with recombinant Vg1 protein (lane 1). Note that the samples in Fig. 3A and B do not resolve a doublet of Vg1 protein that can often be seen on lower percentage gels (see Figs 3C and 5).

By analogy to TGF-β we expect to find a smaller peptide of $M_r = \sim 13{\cdot}1 \times 10^3$ to be cleaved from the full-length Vg1 protein (see Fig. 1), however, we are usually unable to detect such a product by immunoprecipitation (Fig. 3B). We sometimes observe a very weak band at $M_r = \sim 17 \times 10^3$ which may represent the cleaved form of Vg1 protein (see the results of mRNA injection described experiments below).

The immunoprecipitation experiments described

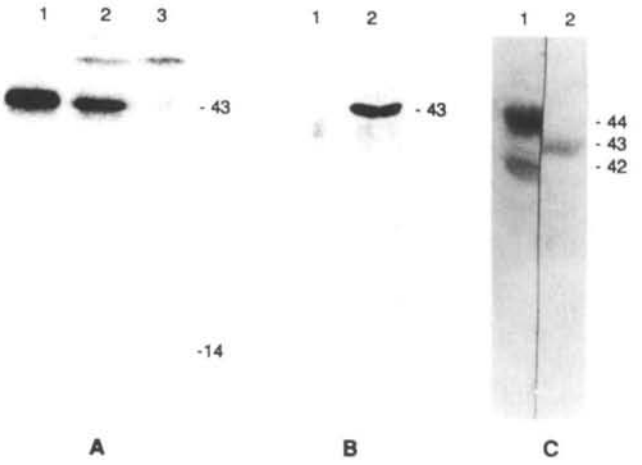

Fig. 3. Anti-Vg1 antibodies recognize endogenous Vg1 protein. (A) ^{35}S-labelled blastula proteins were immunoprecipitated with a monoclonal anti-Vg1 antibody (lane 1) and an anti-Vg1 polyclonal antisera (lane 2) and preimmune antisera (lane 3). This 15 % gel does not resolve the doublet of Vg1 protein that is often seen. Note that no protein band of $M_r = \sim 13 \times 10^3$ can be seen at the predicted size of the secreted Vg1 protein. (B) Immunoprecipitation of the Vg1 protein of $M_r = \sim 43 \times 10^3$ by anti-Vg1 antibodies is eliminated by preincubation of the antibodies with recombinant Vg1 protein (lane 1). Lane 2 is Vg1 protein immunoprecipitated without preincubation with recombinant protein. (C) A lane of a western blot was halved and probed with either an anti-Vg1 monoclonal antibody (left half) or an anti-actin monoclonal antibody (right half). The anti-Vg1 antibody recognizes two proteins of $M_r = \sim 42$ and $\sim 44 \times 10^3$ which are resolved on this 10 % gel, neither of which are actin ($M_r = \sim 43 \times 10^3$). Again, no small Vg1 peptide ($M_r = \sim 13 \times 10^3$) can be detected (but see Fig. 6A). Relative molecular mass ($\times 10^{-3}$) is indicated on the right-hand side.

above analyze the Vg1 protein that is synthesized embryonically, i.e. made after fertilization. By performing western blot experiments on unlabeled proteins we have analyzed total Vg1 protein (both maternally inherited and embryonically synthesized). Fig. 3C shows a Western blot of early *Xenopus* blastula proteins probed with an anti-Vg1 monoclonal antibody (the polyclonal anti-Vg1 antibodies react only weakly in this assay). The blot was split into two and the left half was probed with anti-Vg1 antibody and the right half with anti-actin antibody. This shows that the anti-Vg1 antibody recognizes two bands of $M_r = \sim 42 \times 10^3$ and 44×10^3. Both of these are close to the predicted size of the Vg1 protein and suggests a post-translational modification of the Vg1 protein given that the *in vitro* translated Vg1 protein is some $M_r = \sim 8 \times 10^3$ smaller in size (Figs 2C and 4). The anti-Vg1 antibody does not react with actin which is an abundant protein of a similar molecular weight.

To estimate the amount of Vg1 protein in early blastula, known amounts of recombinant Vg1 protein and early blastula proteins were compared by Western blotting using an anti-Vg1 monoclonal antibody. We estimate that a single early blastula contains approximately 0·5–1·0 ng of full-length Vg1 protein (data not shown).

Processing of Vg1 protein in vitro

To study how Vg1 protein is processed, synthetic Vg1 mRNA was translated *in vitro* in either the presence or absence of canine microsomal membranes, and the products analyzed by immunoprecipitation. The results in Fig. 4 show that a protein of $M_r = \sim 35 \times 10^3$ is translated from Vg1 mRNA in the absence of microsomes (lane 2). This is about $M_r = 8 \times 10^3$ smaller than the predicted relative molecular mass of Vg1 protein, therefore full-length Vg1 protein has a faster than expected mobility on SDS–PAGE. A protein of $M_r = \sim 31 \times 10^3$ is also translated *in vitro* from Vg1 mRNA (Fig 4, lane 2). This protein arises from downstream initiation of translation that occurs *in vitro* (data not shown). Translation of Vg1 mRNA in the presence of microsomes results in a number of slower mobility forms which indicate processing (e.g. glycosylation) of the Vg1 protein (lane 4). These slower mobility forms of Vg1 protein are secreted into the lumen of the membranes as shown by their resistance to proteinase K degradation (lane 5) which is abolished by the addition of detergent (lane 6). Endoglycosidase H (endo H) treatment of Vg1 protein translated in the presence of microsomes converts it to a protein of relative molecular mass similar to that of the unprocessed Vg1 protein (lane 11). These data show that Vg1 is glycosylated at *N*-linked sites.

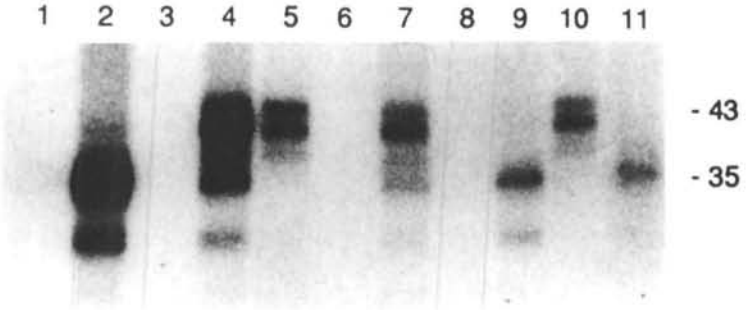

Fig. 4. Vg1 protein is secreted and glycosylated by microsomes *in vitro*. Vg1 mRNA was translated with rabbit reticulocyte lysates in the presence or absence of canine pancreatic microsomal membranes and the translated products were analyzed by immunoprecipitation. Translation of Vg1 in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of microsomes and immunoprecipitated with preimmune (lanes 1 and 3) or anti-Vg1 sera (lanes 2 and 4). Proteinase K treatment of the Vg1 translation products with microsomes in the absence (lane 5) or presence of detergent (lane 6), and immunoprecipitation with anti-Vg1 sera. Alkaline bicarbonate treatment of Vg1 translation products with microsomes fractionated into cytosol (lane 7) and membrane fractions (lane 8). Controls for cytosol (lane 9) and membrane fractions (lane 10) without alkaline bicarbonate treatment. Lane 11 shows endo H treatment of the Vg1 translation products in the presence of microsomes immunoprecipitated with anti-Vg1 antibody. Proteins were separated on a 10% gel. Relative molecular mass ($\times 10^{-3}$) is indicated on the right.

The results described above do not distinguish whether Vg1 is an integral membrane protein or a secreted protein. To address this issue the Vg1 protein translated in the presence of microsomes was treated with or without alkaline bicarbonate and the membrane and cytosol fractions were assayed for Vg1 protein. Alkaline bicarbonate treatment opens microsomes and allows release of their contents into the cytosol (Teixidó *et al.* 1987), thus secreted Vg1 should now be found in the cytosol. If Vg1 was a membrane protein it should remain with the membrane fraction regardless of alkaline bicarbonate treatment. As shown in Fig. 4 (lanes 7 and 8), the Vg1 protein translated in the presence of microsomes is found entirely in the cytosol fraction after treatment with alkaline bicarbonate buffer. This shows that glycosylated Vg1 protein is secreted and has presumably undergone signal sequence removal. Fractionation in the absence of alkaline bicarbonate treatment results in the association of the glycosylated forms of Vg1 with the membranes (lane 10), while the unglycosylated forms remain in the cytosol (lane 9) as expected. (Note that some mRNA is translated on free ribosomes as well as membrane-bound ribosomes in this system.) These experiments show that Vg1 is a glycoprotein that can be secreted into the lumen of the endoplasmic reticulum.

Processing of Vg1 protein in vivo

Although the above experiments show glycosylation and secretion of Vg1 protein *in vitro*, this does not provide direct evidence on how the Vg1 protein is processed *in vivo*. To characterize the Vg1 protein in embryos, we analyzed the endogenous Vg1 protein and also the Vg1 protein made in embryos injected with synthetic Vg1 mRNA. Vg1 protein was immunoprecipitated from stage 8 embryos previously injected with [^{35}S]-Trans-label and the immunoprecipitates treated

with endo F or endo H. Endo H cleaves high-mannose glycans from *N*-linked glycoproteins whereas endo F cleaves both high-mannose and complex glycans from *N*-linked glycoproteins (Elder & Alexander, 1982). As shown in Fig. 5, endo F (lane 2) and endo H (lane 3) treatment converts the endogenous Vg1 protein to faster mobility forms relative to the untreated sample (lane 1). This proves that embryonically synthesized Vg1 protein has *N*-linked glycosylation. A further proof of the *in vivo* glycosylation of Vg1 protein is given by the observation that Vg1 protein made in embryos injected with Vg1 mRNA (lane 4) also shows a comparable sensitivity to endo F (lane 5) and endo H (lane 6). Vg1 protein made in embryos from injected mRNA is therefore processed similarly to endogenous Vg1 protein. Maternal Vg1 protein, detected by Western blotting, is also likely to be glycosylated since it has the same relative molecular mass as the embryonically synthesized Vg1 protein.

Since Vg1 protein is glycosylated *in vivo*, it is likely that it is present in the secretory pathway. Fig. 5 shows the result of an experiment where labelled early blastula proteins are separated into membrane and cytosol fractions and assayed for Vg1 protein by immunoprecipitation. As shown, there is Vg1 protein present in the membrane fraction (lane 7). Again, when Vg1 mRNA is injected into embryos, the resulting Vg1 protein behaves in a manner identical to that of the endogenous protein. The presence of some Vg1 protein in the cytosol fraction may be due to rupturing of the membranes during isolation. Alternatively, it is possible the Vg1 protein has been secreted into the extracellular space. The faster mobility form detected in these experiments is enriched in the cytosol fraction relative to the membrane fraction and perhaps represents an extracellular form of the protein. Overall, these results confirm that the endogenous Vg1 protein is a glycoprotein and that it is associated with cell membranes, presumably the endoplasmic reticulum.

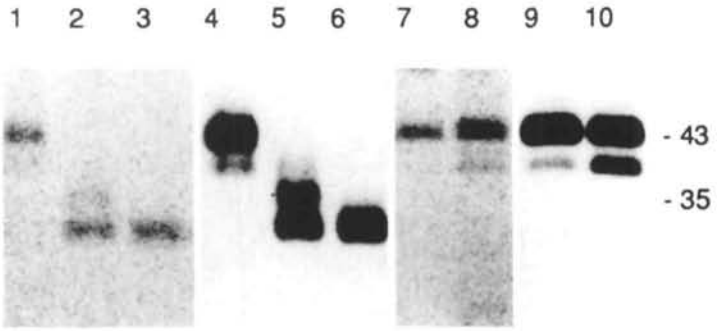

Fig. 5. Vg1 protein is glycosylated and associated with membranes *in vivo*. The glycosylation of Vg1 protein *in vivo* is demonstrated in lanes in 1–6. Immunoprecipitated Vg1 protein, from ^{35}S-labelled blastula embryos, incubated in buffer alone (lane 1) or treated with endo F (lane 2) and endo H (lane 3). The same analysis but from embryos injected with Vg1 mRNA: immunoprecipitates incubated in buffer alone (lane 4) or treated with endo F (lane 5) and endo H (lane 6). The subcellular fractionation of Vg1 protein *in vivo* is shown in lanes 7–10. Endogenous Vg1 protein was immunoprecipitated from either the membrane (lane 7) or the cytosol (lane 8) fraction of ^{35}S-labelled embryos. Similarly, lanes 9–10 show an identical experiment but from embryos injected with Vg1 mRNA. Vg1 protein was immunoprecipitated from either the membrane (lane 9) or the cytosol (lane 10) fraction. Proteins were separated on a 10% gel. Relative molecular mass ($\times 10^{-3}$) is indicated on the right.

The experiments described above support the hypothesis that full-length Vg1 protein ($M_r = \sim 42{\cdot}1 \times 10^3$) can be secreted from a cell, but as shown in Fig. 3 we do not observe significant amounts of a small cleaved Vg1 protein ($M_r = \sim 13{\cdot}1 \times 10^3$). Possibly, we cannot easily detect the small Vg1 protein because it is only made at very low levels relative to its large precursor. Therefore, we increased the total amount of Vg1 protein made by injecting synthetic Vg1 mRNA into fertilized eggs. Injected eggs were allowed to develop until the gastrula stage before analyzing for Vg1 protein by Western blotting. Fig. 6 shows that there is at least a 10-fold increase in the amount of full-length Vg1 protein ($M_r = \sim 43 \times 10^3$) in Vg1 mRNA injected embryos. A small protein of $M_r = \sim 17 \times 10^3$ is also detected at a low level in embryos injected with Vg1 mRNA and thus arises by cleavage of the full-length Vg1 protein. Immunoprecipitation experiments from many uninjected embryos have also shown that a protein of $M_r = \sim 17 \times 10^3$ can be sometimes detected at extremely low levels, which is difficult to photograph (data not shown). Since the small Vg1 protein has the potential for *N*-linked glycosylation, this might explain the size discrepancy between the predicted small Vg1 protein ($M_r = \sim 13 \times 10^3$) and the small Vg1 protein ($M_r = \sim 17 \times 10^3$) seen in injected embryos. Consistent with this is an observation that a protein of $M_r = \sim 17 \times 10^3$ that is immunoprecipitated from Vg1 injected embryos can be converted to a smaller protein of $M_r = \sim 13 \times 10^3$ by endo H treatment (data not shown).

In embryos injected with Vg1 mRNA, the amount of the small Vg1 protein ($M_r = \sim 17 \times 10^3$) detected by Western blotting is at least 20 times less abundant relative to the full-length Vg1 protein ($M_r = \sim 43 \times 10^3$). By analogy, if only 5% of the full-length endogenous Vg1 protein were processed to the small product then it would be beyond the level of detection by Western blot analysis (which is around 0·4 ng in our hands).

Oocytes are known to be efficient secretory cells; therefore, we decided to examine whether oocytes injected with Vg1 mRNA could secrete Vg1 protein. Oocytes injected with Vg1 mRNA were labelled overnight before fractionation of the proteins and analysis by immunoprecipitation. Fig. 6B shows that a large amount of Vg1 protein ($M_r = \sim 43 \times 10^3$) is found in both the membrane fraction (M) and cytosol fraction (C) of oocytes injected with Vg1 mRNA; however, no Vg1 protein is found in the culture media (S). These results are obtained whether or not oocytes have had their follicle cells removed (data not shown). The lack of secreted Vg1 protein in the oocyte culture media is not due to inadequate recovery of material from the media since we can easily obtain secreted interferon

from the media of oocytes injected with interferon mRNA (Fig. 6C). Hence these results indicate that an oocyte does not secrete Vg1 protein.

Expression of Vg1 protein during oogenesis and embryonic development

To examine the expression of the Vg1 protein during oogenesis and early development, a monoclonal anti-Vg1 antibody was used to probe Western blots of different developmental stages (Fig. 7). The synthesis of Vg1 protein is first detected at low levels in stage IV oocytes (this does not reproduce well in the photograph) and then increases throughout oogenesis and maturation. Vg1 protein cannot be found before stage IV of oogenesis, even when more oocyte equivalents are loaded on the gel (data not shown). Therefore, it appears that the majority of Vg1 protein is synthesized during oogenesis after the mRNA has become localized to the vegetal pole (Melton, 1987*a*; Yisraeli & Melton, 1988).

Fig. 7B shows the expression of total (maternally

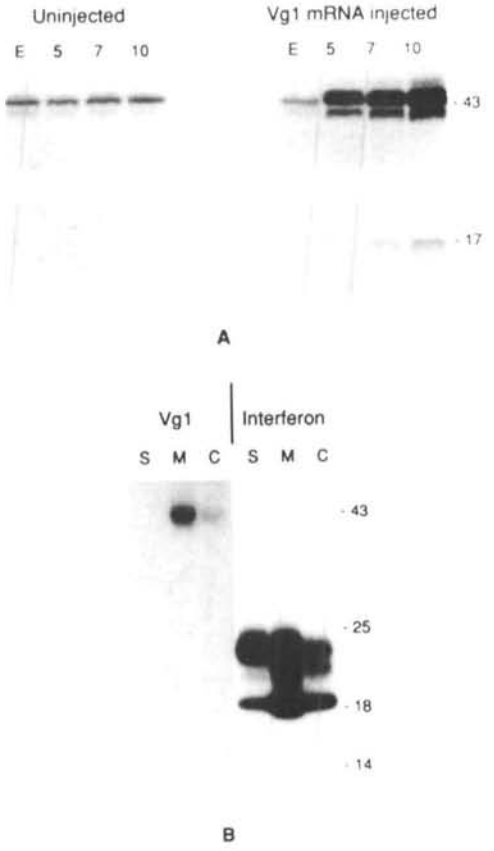

Fig. 6. A small Vg1 protein exists in embryos injected with Vg1 mRNA, though Vg1 protein is not secreted from oocytes injected with Vg1 mRNA. (A) Western blot of uninjected embryos or embryos injected at the one cell stage with Vg1 mRNA and analyzed at the stages indicated with a monoclonal anti-Vg1 antibody (E = fertilized egg). Relative to uninjected embryos, the amount of large Vg1 protein ($M_r = \sim 43 \times 10^3$) increases in the Vg1 mRNA injected embryos as they develop. In the injected embryos, a smaller protein ($M_r = \sim 17 \times 10^3$) also accumulates as development proceeds. Proteins were separated on a 10% gel (top panel) and 15% gel (bottom panel).
(B) Immunoprecipitation of Vg1 protein from ^{35}S-labelled oocytes injected with Vg1 mRNA (left panel). No Vg1 protein can be detected in the culture supernatant (S), but large Vg1 protein ($M_r = \sim 43 \times 10^3$) is found in both the membrane (M) and cytosol (C) fractions. The right panel shows that oocytes injected with interferon mRNA secrete interferon protein into the supernatant (S), as detected by immunoprecipitation with an anti-interferon antibody. Proteins were separated on a 15% gel. Relative molecular mass ($\times 10^{-3}$) is indicated on the right.

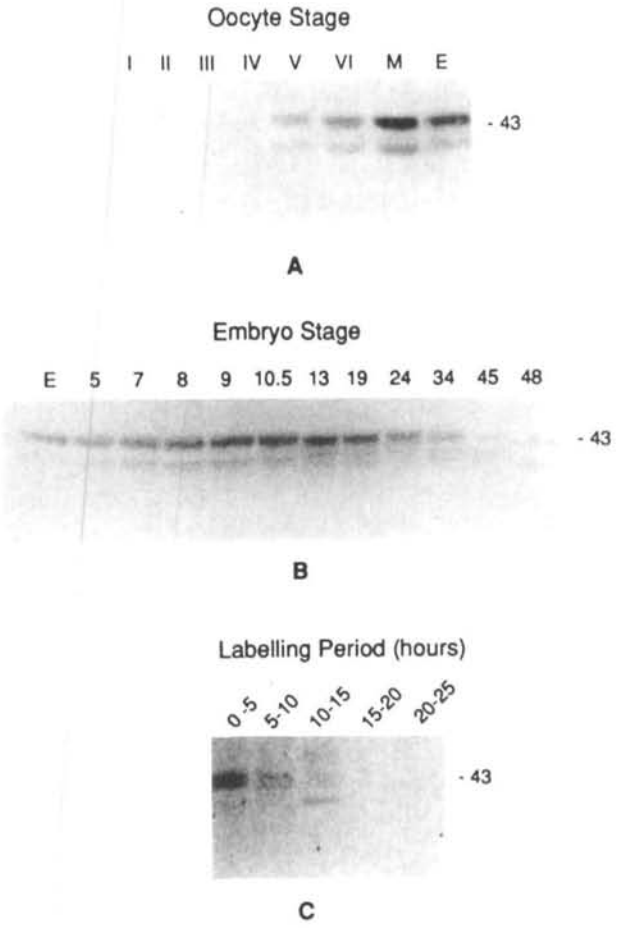

Fig. 7. The expression of Vg1 protein during oogenesis and embryonic development. (A) Western blot of different stages of oogenesis probed with a monoclonal anti-Vg1 antibody. Oocyte stages are according to Dumont (1972). M represents stage VI oocytes matured by progesterone treatment and E represents a fertilized egg. (B) Western blot of different stages of embryonic development probed with a monoclonal anti-Vg1 antibody. Embryo stages are according to Nieuwkoop & Faber (1967). E represents a fertilized egg. (C) Analysis of Vg1 protein synthesis during early development. Vg1 protein was immunoprecipitated from ^{35}S-labelled embryos pulse-labelled for different 5 h periods as indicated. The labelling periods correspond to stages of development as follows: 0–5 h is from fertilized egg to 64/128 cell stage; 5–10 h is from 64/128 cells to early gastrula, 10–15 h is from early gastrula to early neurula, 15–20 h is from early neurula to late neurula and 20–25 h is from late neurula to early tailbud. Proteins were separated on a 10% gel. Relative molecular mass ($\times 10^{-3}$) is indicated on the right-hand side.

inherited plus embryonically synthesized) Vg1 protein during early embryonic development. From the newly fertilized egg until the early gastrula, there is a slight increase in the amount of Vg1 protein present. From neurula to swimming tadpole, there is a significant decrease in protein detected with the anti-Vg1 antibody. Given the observation that the majority of Vg1 mRNA is degraded during gastrulation (Rebagliati *et al.* 1985), this suggests that Vg1 protein is rather stable.

To examine when Vg1 protein is synthesized during embryogenesis, embryos were injected with label at different developmental stages and allowed to develop for 5 h before analyzing for Vg1 protein by immunoprecipitation. As shown in Fig. 7C, the majority of Vg1 protein is made prior to the gastrula stage. Each consecutive labelling period has less Vg1 protein than the previous one, and no Vg1 protein appears to be made after neurulation. These results are consistent with the fact that most of the Vg1 mRNA is degraded by the late gastrula stage (Rebagliati *et al.* 1985).

Vegetal localization of the Vg1 protein during early development

One important feature of mesoderm induction is that the signal(s) for induction emanate from the vegetal hemisphere of the egg. The results presented in Fig. 8 show that Vg1 protein is localized in the vegetal hemisphere of eggs and blastula. Embryos were injected with label at the 1-cell stage and allowed to develop until either the 64-cell stage or until a stage-8 blastula before dissection into animal and vegetal halves and subsequent analysis for Vg1 protein by immunoprecipitation (Fig. 8A, left-hand 6 lanes). The Vg1 protein made between the 64-cell stage and stage 8 was similarly analyzed, by injecting label into the 64-cell embryo (right-hand 3 lanes). As shown, the embryonically synthesized Vg1 protein made during both early and later cleavage is confined to the vegetal pole. These results are in keeping with the vegetal localization of Vg1 mRNA (Rebagliati *et al.* 1985; Weeks & Melton, 1987).

Although the embryonically synthesized Vg1 protein is localized to the vegetal hemisphere, it is possible that maternally inherited Vg1 protein has a uniform distribution within the embryo. Fig. 8B shows a Western blot assaying the distribution of total Vg1 protein in the fertilized egg, 64-cell blastula and the stage-8 blastula. Two Vg1 protein bands are detected: the more abundant Vg1 protein ($M_r = \sim 44 \times 10^3$) is localized to the vegetal pole in all these stages, and the less abundant Vg1 protein ($M_r = \sim 42 \times 10^3$) is more uniformly distributed. Also shown below is an identical blot probed with an anti-actin antibody, showing that there are approximately similar amounts of protein in each of the dissected embryo halves. The uniformly distributed form of Vg1 protein ($M_r = \sim 42 \times 10^3$), present in the animal hemisphere, is likely to be maternal in origin since embryonically synthesized Vg1 protein is found only in the vegetal pole. It is possible that only one these large Vg1 proteins can be processed and/or secreted, but at present, we have no data on differential processing.

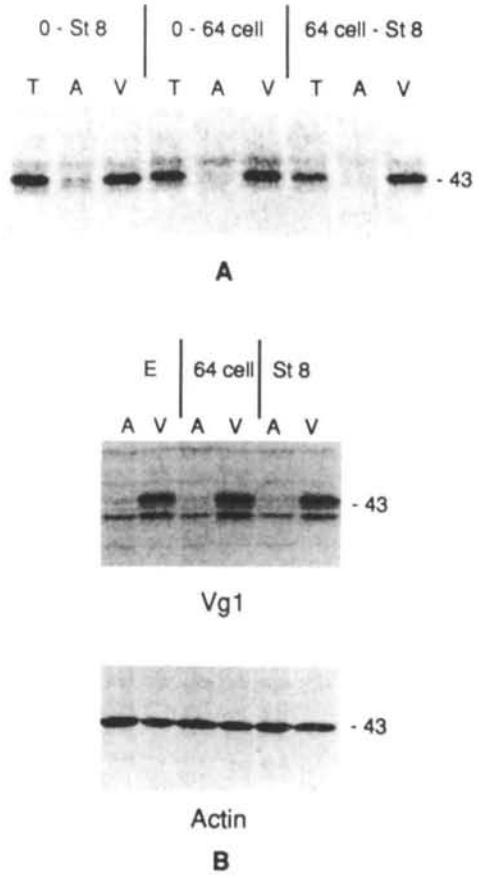

Fig. 8. Vg1 protein is localized to the vegetal hemisphere of early embryos. (A) Embryonically synthesized Vg1 protein is vegetally localized in embryos. Fertilized eggs (left and middle panels) or 64-cell-stage embryos (right panel) were injected with ^{35}S-trans-label and allowed to develop to the 64-cell or mid-blastula stage, respectively, before dissection into animal and vegetal halves. Vg1 protein was detected by immunoprecipitation using anti-Vg1 antibody. T represents total undissected embryos and A and V represents equal numbers (10) of dissected animal and vegetal halves, respectively. The uniformly distributed band present just above the Vg1 protein bands is a non-specific background band that also appears in the preimmune control (not shown). Proteins were separated on a 15% gel. (B) Total Vg1 protein (comprising both maternally inherited and embryonically synthesized) is also vegetally localized in embryos to the vegetal pole. Embryos were dissected into animal and vegetal halves at the stages indicated. Isolated proteins were western blotted and probed with a monoclonal anti-Vg1 antibody. E represents a fertilized egg. Also shown, in the lower panel, is a similar blot which has been probed with anti-actin monoclonal antibody. A and V again represent equal numbers (10) of dissected animal and vegetal halves, respectively. Proteins were separated on a 10% gel. Relative molecular mass ($\times 10^{-3}$) is indicated on the right.

Discussion

We have previously proposed that the Vg1 protein is a

natural mesoderm-inducing molecule since the mRNA is localized to the vegetal pole and encodes a protein with sequence similarity to known inducers (Weeks & Melton, 1987). In this paper, we show that the Vg1 protein is synthesized before gastrulation only in the vegetal hemisphere of the early blastula. In addition, we have shown that the Vg1 protein is glycosylated and associated with the membrane fraction of embryos and that it can be secreted into the lumen of pancreatic microsomes. Hence, Vg1 protein is synthesized both at the time when embryos are known to be competent for mesoderm induction (Dale *et al.* 1985; Gurdon *et al.* 1985; Jones & Woodland, 1987; Nakamura *et al.* 1971), and in the place where induction signals are known to arise (Gurdon, 1989; Gurdon *et al.* 1985; Nakamura *et al.* 1971; Nieuwkoop, 1969; Smith *et al.* 1985). These results are consistent with, but do not prove, the hypothesis that Vg1 is indeed an endogenous mesoderm-inducing factor.

By analogy to TGF-β, a small secreted form of Vg1 protein is likely to be the active molecule. Currently, we have only indirect evidence that the endogenous Vg1 protein is processed to yield a small protein ($M_r = \sim 17 \times 10^3$) that is secreted. Our *in vitro* studies on glycosylation and secretion suggest that the endogenous protein will be similarly processed. In addition, Vg1 mRNA injections provide strong evidence for cleavage of the full-length Vg1 protein into a small 'growth factor'-size molecule. It is possible that we do not reproducibly detect a small secreted form of the endogenous Vg1 protein because it is generated in low amounts and/or is rapidly turned over. However, we do see a low amount of cleaved Vg1 protein ($M_r = \sim 17 \times 10^3$) in embryos injected with Vg1 mRNA, although we do not know if it is secreted. If the amount of non-yolk protein in a blastula is taken as 25 μg and the volume as 1·0 μl (Gurdon & Wickens, 1983), this means the endogenous large Vg1 protein ($M_r = \sim 43 \times 10^3$) is present at a concentration of 0·5–1·0 $\mu g\,ml^{-1}$ (10–20 nM) within a blastula. However, if a small secreted Vg1 protein was produced at a level 10- to 20-fold times less than its large precursor, then the concentration of a small secreted Vg1 protein might be similar to the concentrations at which other growth factors have effects on *Xenopus* embryos. In all, our data highlight the fact that the expression of the gene is regulated in several ways: localization of mRNA and protein, cleavage and glycosylation of full-length protein and secretion.

While we have tested for glycosylation and secretion of the Vg1 protein, it is possible that other post-translational controls exist in generating active Vg1 product. For example, it is known that TGF-βs are made in latent forms which can be activated by protease treatment or low pH (Lyons *et al.* 1988; Miyazono *et al.* 1988; Wakefield *et al.* 1988). Recently, it has also been shown that endo F can also activate TGF-β1 (Miyazono & Heldin, 1989). It is thus interesting that Vg1 is sensitive to both endo F and endo H. Similarities to the TGF-β family suggest that Vg1 would function as a dimer, therefore the formation of either homodimers or heterodimers to yield active Vg1 protein may also be regulated.

Vg1 protein is first detected in midstage oocytes but the maximal levels are found in larger oocytes. Therefore the localization of Vg1 mRNA into a tight subcortical shell (Melton, 1987*a*; Yisraeli & Melton, 1988) does not interfere with the translation of Vg1 protein. Injected Vg1 mRNA is also translated in oocytes, but none of the Vg1 protein synthesized is secreted. Since oocytes can secrete a large number of proteins, this suggests a potentially interesting control mechanism whereby Vg1 protein secretion is tightly regulated.

During early development we can detect synthesis of new Vg1 protein until the gastrula stage. This embryonically synthesized Vg1 protein is found only in the vegetal hemisphere of blastula. Total (maternally inherited plus embryonically synthesized) Vg1 protein is also largely confined to the vegetal hemisphere of blastula although a maternally inherited component ($M_r = \sim 42 \times 10^3$) is also present in the animal hemisphere. While the Vg1 protein is present in the early embryo at a time and place consistent with acting in mesoderm induction, it is noted that the Vg1 protein persists long after mesoderm induction is complete. Presently, we do not have any data that suggest a function for this persistent portion of the Vg1 gene product.

Recently, Dale *et al.* (1989) have independently characterized the Vg1 protein by immunoprecipitation using anti-Vg1 antibodies. Although essentially similar, our results differ from Dale *et al.* in several respects. Since our monoclonal anti-Vg1 antibodies work in Western blots, this has allowed us to show that Vg1 protein is rather stable throughout development. More importantly, we have provided evidence that the Vg1 protein is processed into a small peptide ($M_r = \sim 17 \times 10^3$) by overexpressing Vg1 in embryos. Finally, Fig. 8B shows that the egg inherits some of the faster mobility form of Vg1 protein ($M_r = \sim 42 \times 10^3$) in the animal pole. Considering the observation of Dale *et al.* that some Vg1 protein can diffuse into the animal pole of oocytes, this suggests that diffusion of the larger Vg1 protein ($M_r = \sim 44 \times 10^3$, see Fig. 8B) into the animal pole of eggs is restricted or that it is relatively unstable in the animal pole.

The dorsal mesoderm-inducing signal may involve a molecule related to TGF-β, given that TGF-β1 acts synergistically with bFGF to give large amounts of muscle (Kimelman & Kirschner, 1987) and given that the TGF-β-like factor XTC-MIF can induce dorsal mesoderm (Smith, 1987). The dorsal region of the vegetal hemisphere releases the dorsal-inducing signal(s) (Boterenbrood & Nieuwkoop, 1973; Dale & Slack, 1987; Dale *et al.* 1985). Therefore, these observations, together with the vegetal localization of Vg1 protein and sequence similarity of Vg1 to TGF-β, raise the possibility that Vg1 acts alone or together with bFGF to induce dorsal mesoderm. Indeed, preliminary results suggest that Vg1 can act synergistically with bFGF to induce mesoderm. Further work is in progress to characterize this effect in more detail. If Vg1 is

involved in inducing dorsal or dorsal/anterior mesoderm, then the Vg1 protein may be synthesized or processed differentially in dorsal *versus* ventral blastomeres.

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