The EMBO Journal vol.8 no.4 pp.1057 - 1065, 1989

Developmental expression of the protein product of Vg1, a localized maternal mRNA in the frog *Xenopus laevis*

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Communicated by T.Hunt

Vg1 is a maternal mRNA localized in the vegetal cortex of *Xenopus laevis* oocytes, that encodes a protein homologous to the mammalian growth factor TGF-β. Using a polyclonal antibody to a T7–Vg1 fusion protein, we have identified the native protein. We find that a single protein of M_r 40 kd is immunoprecipitated following *in vitro* translation of oocyte poly(A)$^+$ RNA, whilst two proteins of M_r 45 and 43.5 kd are immunoprecipitated from oocyte and embryo extracts. Synthesis of at least the 40 kd, *in vitro*, and 45 kd, *in vivo*, proteins is specifically inhibited following treatment of the respective systems with antisense Vg1 (but not histone H4) oligodeoxynucleotides. Tunicamycin treatment reveals the *in vivo* proteins to be glycosylated versions of a 40 kd protein, modified by the addition of either two or three N-linked oligosaccharide side chains. Both proteins are sensitive to digestion by the enzyme endoglycosidase-H, and are segregated within a membrane fraction from which they can be released by high pH treatment. Their synthesis is first detectable in stage IV oocytes and continues throughout early embryogenesis until the late gastrula. During embryogenesis the relative proportions of the two proteins change, the 45 kd protein being predominant in early embryogenesis and the 43.5 kd protein in late embryogenesis. Synthesis only occurs in the vegetal hemisphere at all stages; however, in the large oocyte diffusion of both proteins into the animal hemisphere occurs.

Key words: localized maternal mRNA/TGF-β family/Vg1 protein/*Xenopus* development

Introduction

Two main mechanisms are thought to be responsible for the specification of cell fate in early embryos: cytoplasmic localization and induction (Slack, 1983; Davidson, 1986). In many types of embryo the unequal distribution of different egg cytoplasmic components is believed somehow to determine the fate of those blastomeres that receive them. A large body of evidence supports the existence of these cytoplasmic determinants, and much of this evidence has recently been reviewed by Davidson (1986). For example, the formation of both major axes of the early amphibian embryo, animal-vegetal and dorso-ventral, are believed to be the result of determinants localized during oogenesis and early embryogenesis (Gerhart, 1980, 1987).

There is also a considerable body of evidence that many cell fates are determined by inductive interactions between different regions of the embryo (Spemann, 1938; Nieuwkoop, 1973; Gurdon, 1987). In amphibians, for example, it is clear that all mesodermal tissues are formed in this way (Nieuwkoop, 1969; Dale *et al.*, 1985; Dale and Slack, 1987b). Animal pole cells which in isolation would form epidermis are induced to form mesoderm by the underlying vegetal pole cells. Similarly, dorsal mesoderm induces the overlying ectoderm to form neural tissue (Gimlich and Cooke, 1983; Smith and Slack, 1983). Of course it is clearly possible that a cell's ability to supply or respond to an inductive signal is specified by determinants, localized in the early embryo or during some prior stage of oogenesis.

Although these phenomena have been studied extensively since the beginning of the century, there are few examples of molecules that may be involved in either of these processes. Recently cDNA clones for a class of localized maternal mRNAs have been isolated from eggs of the frog *Xenopus laevis* (Rebagliati *et al.*, 1985). One such mRNA, Vg1, is localized to the vegetal hemisphere of fully grown oocytes and early embryos of this species (Melton, 1987; Weeks and Melton, 1987). Fate mapping studies have shown that this region of the embryo will form endoderm in the tadpole (Dale and Slack, 1987a), a fate maintained when this region is isolated (Nieuwkoop, 1969, 1973; Gerhart, 1980). Furthermore, as mentioned above, this region is known to be the source of factors responsible for the induction of mesoderm during early embryogenesis. During development, therefore, molecules such as Vg1 may act as either localized determinants or inducing factors. Interestingly this mRNA encodes a member of the TGF-β (transforming growth factor β) family of proteins (Weeks and Melton, 1987), a family that has been implicated in many early developmental events (Sporn *et al.*, 1987) including mesoderm induction in amphibians (Kimelman and Kirschner, 1987; Rosa *et al.*, 1988).

We have recently isolated Vg1 cDNA clones which we have used to generate a fusion protein containing much of the C-terminal domain of Vg1, including all of the TGF-β homologous sequences. This fusion protein has been used to raise a polyclonal antibody which immunoprecipitates a 40 kd protein synthesized *in vitro* and both 45 kd and 43.5 kd proteins synthesized *in vivo*. Both *in vivo* proteins are N-glycosylated versions of a 40 kd protein and are associated with membranous compartments of both oocytes and embryos. We find that synthesis of the *in vivo* proteins is first detectable in the stage IV oocyte and continues until the late gastrula stages of embryogenesis. The relative proportions of the two proteins change during embryogenesis, the 45 kd protein being the predominant protein synthesized in early embryos and oocytes and the 43.5 kd protein in later embryos. Both proteins are only synthesized

in the vegetal hemisphere at all stages of development; however, during oogenesis the proteins are able to diffuse into the animal hemisphere.

Results

A polyclonal antibody to Vg1

To analyse the Vg1 product in *Xenopus* development, we have raised rabbit polyclonal antibodies against a T7−Vg1 fusion protein containing the carboxy-terminal 156 amino acids of the putative Vg1 protein. This represents 43% of the predicted Vg1 product and includes all 114 amino acids of the putative TGF-β homologous domain (Weeks and Melton, 1987). When analysed on Western blots these antibodies recognize both the T7−Vg1 fusion protein and a second β-Gal−Vg1 fusion protein, suggesting that the antibodies interact with the putative Vg1 portion of these proteins (data not shown). Using the same procedures the antibody did not cross-react with mature porcine TGF-β1. In addition, when a truncated synthetic Vg1 mRNA and chicken oviduct mRNA were simultaneously translated *in vitro*, only the Vg1 product was immunoprecipitated by the antibody (data not shown, but see Figure 1, track 2).

Ovary Vg1 mRNA is translatable in vitro

Although Vg1 mRNA is a polyadenylated species present in the oocyte, there is no previous evidence attesting to its translatability. In the experiment shown in Figure 1, oocyte poly(A)$^+$ RNA, with and without the addition of synthetic truncated Vg1 mRNA, was translated in the wheat germ translation system and the protein products immunoprecipitated with the anti-Vg1 polyclonal antibody. Apart from the 28 kd truncated product only a single protein of M_r 40 kd was precipitated (Figure 1, tracks 2 and 3), approximating quite closely to the 41.8 kd protein predicted from sequencing Vg1 cDNA (Weeks and Melton, 1987). To confirm that this was indeed Vg1 protein, oocyte poly(A)$^+$ RNA was prehybridized with either Vg1 or histone H4 antisense oligonucleotides and digested with RNase H prior to translation in the wheat germ system. Northern blot analysis confirmed that the Vg1 mRNA was degraded only in the presence of the Vg1 antisense oligonucleotide (data not shown). Since the wheat germ system appears to contain an endogenous RNase H activity (Minshull and Hunt, 1986) oligo-annealed oocyte poly(A)$^+$ RNA was also added without a prior digestion step. Under both experimental conditions Vg1 but not H4 antisense oligonucleotides deleted the immunoprecipitable 40 kd protein (Figure 1, cf. tracks 5 and 7). The oligo treatment had no effect on general protein synthesis in the wheat germ system, as determined by TCA precipitable counts and SDS−PAGE of total translation products, when compared to control untreated oocyte mRNA (data not shown). These data indicate that Vg1 protein sequences can be specifically precipitated by the anti-fusion protein antibodies. They also demonstrate that oocyte Vg1 mRNA is translatable.

Vg1 mRNA is translated in both oocytes and embryos

Initially we attempted to identify the protein in extracts of *Xenopus* embryos separated by SDS−PAGE and analysed by Western blot. However, under these conditions we failed to identify any specific proteins with the antibodies. Whilst the reason for the lack of detection might be of a technical

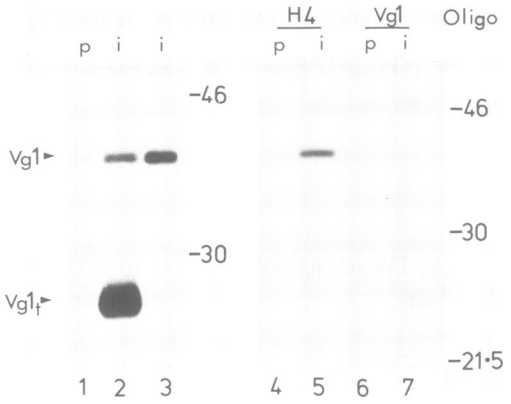

Fig. 1. *In vitro* translation of oocyte poly(A)$^+$ RNA. Oocyte poly(A)$^+$ RNA was translated in a wheat germ translation system containing [^{35}S]methionine for 1.5 h and then immunoprecipitated with either preimmune (p) or anti-Vg1 immune (i) serum. In **tracks 1** and **2** a synthetic and truncated Vg1 mRNA ($Vg1_t$) was also included in the translation. For **tracks 4−7** the RNA was prehybridized with either histone H4 (**tracks 4** and **5**) or Vg1 (**tracks 6** and **7**) antisense oligodeoxynucleotides. The positions of mol. wt markers are indicated.

nature it might also be because Vg1 protein is present at steady-state levels too low to be detected by this method.

We therefore used immunoprecipitation of ^{35}S-labelled oocyte and embryo extracts as a means of identifying the Vg1 protein product *in vivo*. This procedure identified two Vg1 specific proteins in both stage VI oocyte and early blastula embryo extracts, a major protein of M_r 45 kd and a minor one of M_r 43.5 kd (Figure 2A). However, in other experiments performed on the oocyte this latter protein is often not detected.

To confirm that these proteins were indeed the translation products of Vg1 mRNA, stage VI oocytes were injected with antisense oligonucleotides to either Vg1 or histone H4 under conditions which are known to ablate most of the respective endogenous mRNAs (Shuttleworth and Colman, 1988; Shuttleworth *et al.*, 1988). When oocytes were injected with antisense oligonucleotides to histone H4 normal levels of the 45 kd protein (the 43.5 kd protein could not be detected in this experiment) were detected (Figure 2B, track 4). However, we could detect very little of this protein in oocytes injected with antisense oligonucleotides to Vg1 (Figure 2B, track 2). As with the *in vitro* studies described above these oligonucleotides appear to have no effect on overall protein synthesis in the oocyte as measured by TCA precipitable counts and SDS−PAGE of total labelled proteins (data not shown).

Identification of Vg1 protein in staged oocyte and embryo extracts

Radiolabelled extracts from a staged series of oocytes and embryos were immunoprecipitated with both preimmune and Vg1 antibodies. During oogenesis Vg1 protein was found from stage IV onwards (Figure 3A), including oocytes matured by the addition of progesterone to the culture medium (data not shown). Synthesis at earlier stages was never detected. Newly synthesized Vg1 protein can be detected throughout early embryogenesis including gastrulae (Figure 3B). As expected, the protein is not synthesized in neurulae (data not shown), when the mRNA is no longer detected (Rebagliati *et al.*, 1985). However, in late blastulae

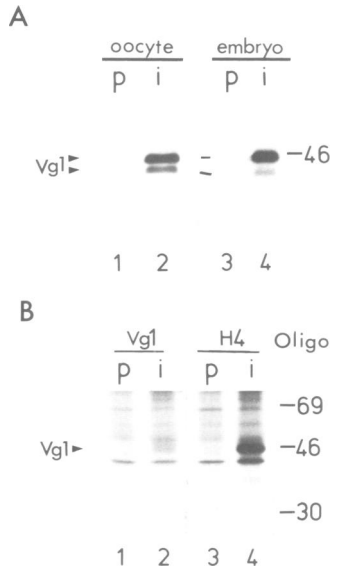

Fig. 2. *In vivo* detection of Vg1 protein. (**A**) Stage VI oocytes (**tracks 1** and **2**) and two-cell embryos (**tracks 3** and **4**) were labelled with [^{35}S]methionine for either 18 h (oocytes) or 2 h (embryos). Homogenates were then immunoprecipitated as in Figure 1. Two proteins of M_r 45 kd and 43.5 kd were detected. (**B**) Stage VI oocytes were injected with either histone H4 (**tracks 1** and **2**) or Vg1 (**tracks 3** and **4**) antisense oligodeoxynucleotides and incubated overnight prior to labelling for 6 h in [^{35}S]methionine. Homogenates were then immunoprecipitated as in Figure 1. In this particular experiment only the 45 kd protein could be detected and then only in oocytes injected with the H4 antisense oligonucleotide.

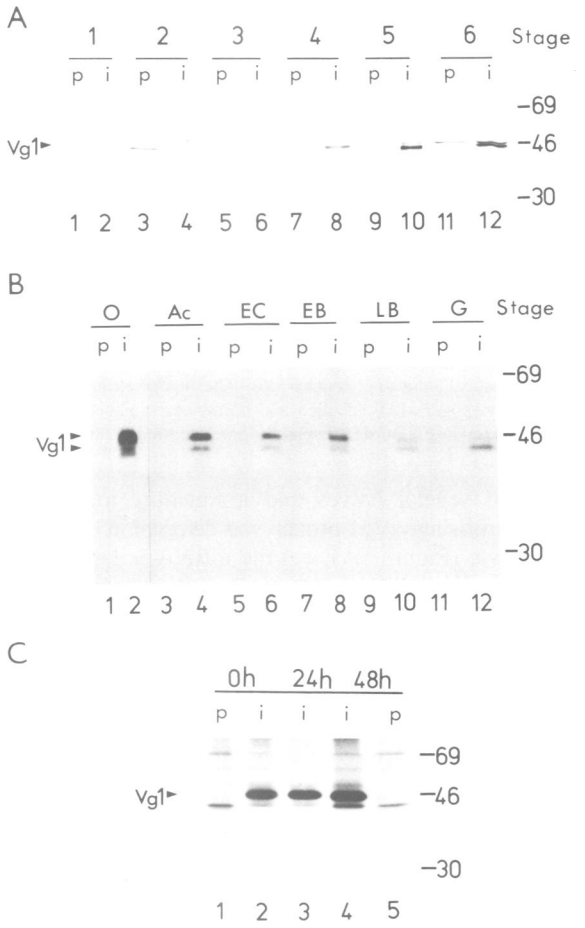

Fig. 3. Developmental expression of Vg1 proteins. (**A**) Oocytes from stages 1–6 were radiolabelled and immunoprecipitated as in Figure 2. Only the 45 kd protein was detected in this experiment. The band seen in the preimmune sample of stage 2 oocytes (**track 3**) was present in all preimmune tracks although at lower intensity. (**B**) Embryos were radiolabelled and immunoprecipitated as in Figure 2. O, stage VI oocyte; Ac, activated egg; EC, early cleavage embryo; EB, early blastulae; LB, late blastulae; G, early gastrulae. (**C**) Stage VI oocytes were labelled with [^{35}S]methionine for 24 h (0 h) and then chased with cold methionine for either 24 or 48 h. Homogenates were then immunoprecipitated as in Figure 1.

and early gastrulae the relative proportions of the two major proteins change, the 43.5 kd protein becoming the most prominent at these stages (compare tracks 1–8 with tracks 9–12 of Figure 3B). The significance of this change is not clear, but it appears to be the result of a change in the glycosylation pattern of a single polypeptide (see below).

In stage VI oocytes the protein appears to be very stable: when labelled oocytes were chased in cold methionine for up to 3 days prior to immunoprecipitation of Vg1 protein turnover of the protein appears to be negligible (Figure 3C). The protein is also stable following progesterone-induced maturation (data not shown).

Vg1 is an N-linked glycoprotein

The amino acid sequence of Vg1, predicted from the cDNA sequence (Weeks and Melton, 1987), contains three potential N-linked glycosylation sites located at amino acids 113, 181 and 301. This suggests that the 45 and 43.5 kd proteins may represent a single polypeptide with different degrees of glycosylation. To test this, stage VI oocytes were injected with tunicamycin, a known inhibitor of N-linked glycosylation, and cultured overnight before radiolabelling; previous work has shown that under these conditions N-glycosylation is completely inhibited (Colman *et al.*, 1981). Only a single protein of M_r 40 kd was detected (Figure 4A, track 6), a protein that co-migrated with the *in vitro* Vg1 product (Figure 4A, track 7).

When oocytes are labelled immediately after injection of tunicamycin, oligosaccharide side chain addition is impaired but not completely inhibited (A.Colman, unpublished). With proteins containing more than one N-glycosylated side chain, this allows the generation of glycosylation intermediates possessing fewer side chains. When this experiment was performed we detected four bands of M_r 45, 43.5, 42 and 40 kd (Figure 4A, track 4). This suggests that the 45 and 43.5 kd proteins that we observe during normal development possess three and two N-linked oligosaccharide side chains respectively.

During passage through the medial compartment of the Golgi, the oligosaccharide side chains of many (though not all) N-glycosylated proteins acquire resistance to the enzyme endoglycosidase-H (endo-H) due to a combination of the trimming of mannose residues and the addition of terminal sugars (Kornfeld and Kornfeld, 1985). We have challenged the Vg1 protein produced in oocytes (Figure 4B) and gastrulae (data not shown) with endo-H. Both proteins are completely sensitive, as judged by their comigration with the protein made in the presence of tunicamycin.

Vg1 protein is synthesized in the vegetal hemisphere

The distribution of Vg1 protein along the animal-vegetal axis of Stage VI oocytes was determined by cutting them into

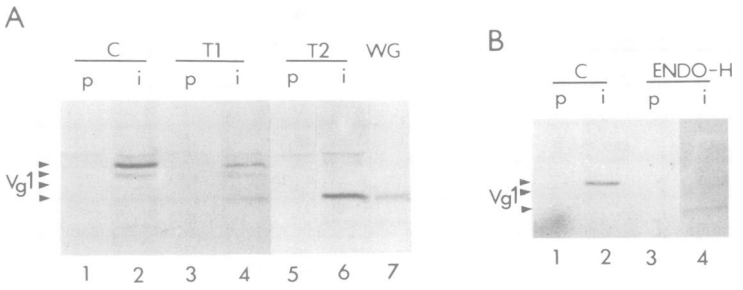

Fig. 4. N-linked glycosylation of Vg1 protein. (**A**) Stage VI oocytes were radiolabelled and immunoprecipitated as described in Figure 2. Oocytes were labelled either without injection of tunicamycin (C; **tracks 1, 2**), or immediately after injection of tunicamycin (T1; **tracks 3,4**) or 18 h after injection of tunicamycin (T2; **tracks 5,6**). The *in vitro* translation product of a full-length synthetic Vg1 mRNA (WG) is shown in **track 7**. (**B**) Stage VI oocytes were radiolabelled and immunoprecipitated as described in Figure 2. Immunoprecipitated protein was then digested with endoglycosidase-H (ENDO-H, **tracks 3,4**), undigested controls are also shown (C, **tracks 1,2**).

animal and vegetal halves and immunoprecipitating the protein. Surprisingly Vg1 protein was detected in both halves of the oocyte (Figure 5A), yet the mRNA is known to be localized to the vegetal hemisphere at this stage (Melton, 1987). Since these oocytes were labelled for 6−18 h it was possible that diffusion of the protein away from a localized translation site could be responsible for this apparent discrepancy. Such diffusion is known to occur following localized translation of exogenous mRNAs in the oocyte (Drummond *et al.*, 1985; Ceriotti and Colman, 1988). To test this we labelled stage VI oocytes for 2.5 h and then bisected them into animal and vegetal halves either immediately after the labelling period, or following a 24-h chase in cold methionine. It is clear from Figure 5B (tracks 1−4) that Vg1 protein is indeed only synthesized in the vegetal hemisphere, but then diffuses into the animal hemisphere (tracks 5−8). Unfortunately we cannot comment further on the spatial distribution of this protein along the animal-vegetal axis since preliminary attempts at immunolocalization have proved unsuccessful.

The distribution of Vg1 protein along the animal-vegetal axis of the embryo was determined by dissecting radiolabelled gastrulae into animal and vegetal halves followed by immunoprecipitation. From this experiment it is clear that Vg1 protein is only synthesized in the vegetal hemisphere and, at least over the incubation period (2 h), does not diffuse to other regions of the embryo (Figure 5C, tracks 3,4). The synthesis of Vg1 protein in the vegetal hemisphere is of course consistent with the known localization of the mRNA to this region (Rebagliati *et al.*, 1985; Weeks and Melton, 1987). The same result was obtained when isolated animal, vegetal and marginal zone fragments were incubated for 3 h in MBS containing [^{35}S]methionine (data not shown).

Although Vg1 mRNA appears to be distributed throughout the vegetal hemisphere of the embryo (Weeks and Melton, 1987), we wished to know if this were true of its protein product. Consequently, embryos labelled during the late blastula stages were divided into dorsal and ventral halves at stage 10 using the dorsal lip as a marker, and Vg1 immunoprecipitated. From the data presented in Figure 5C (tracks 5 and 6) it is clear that at least at this stage Vg1 protein is synthesized in both dorsal and ventral halves. Although the results presented in Figure 5C indicate that more Vg1 was synthesized in dorsal than ventral halves, this difference was not repeated in subsequent experiments. The same result was obtained if dorsal and ventral vegetal poles were labelled for 3 h following dissection at stage 10.

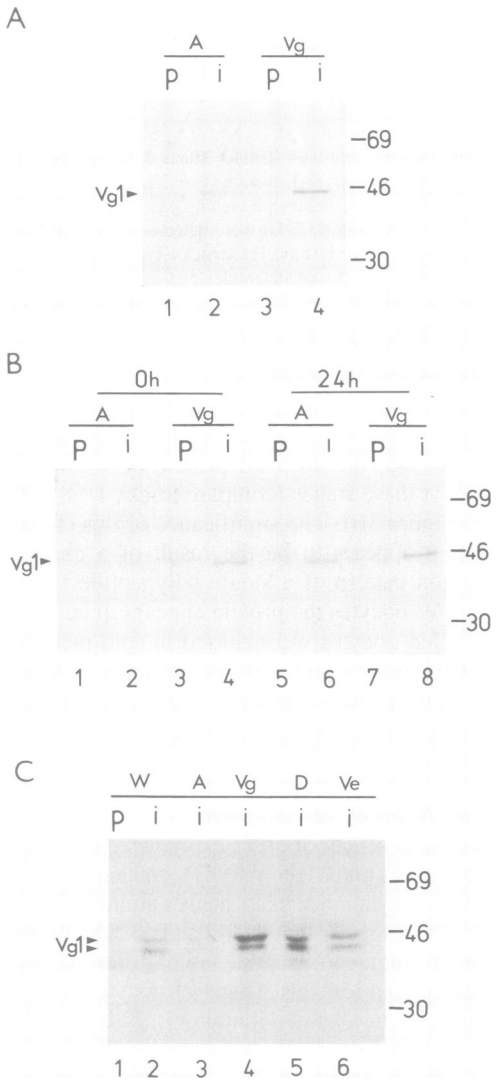

Fig. 5. Localization of Vg1 protein. (**A**) Stage VI oocytes were radiolabelled, bisected into animal (A) and vegetal (Vg) halves and pooled halves immunoprecipitated as in Figure 2. Only the 45 kd protein is visible in this gel. (**B**) Stage VI oocytes were radiolabelled for 2.5 h and then bisected into animal and vegetal halves either before (**tracks 1−4**) or after a 24 h chase with cold methionine (**tracks 5−8**). Only the 45 kd protein is visible in this gel. (**C**) Stage 9 embryos were radiolabelled and immunoprecipitated as in Figure 2 (**tracks 1** and **2**). Alternatively embryos were bisected into either animal (A) and vegetal (Vg; **tracks 3, 4**) or dorsal (D) and ventral (Ve; **tracks 5** and **6**) halves prior to immunoprecipitation.

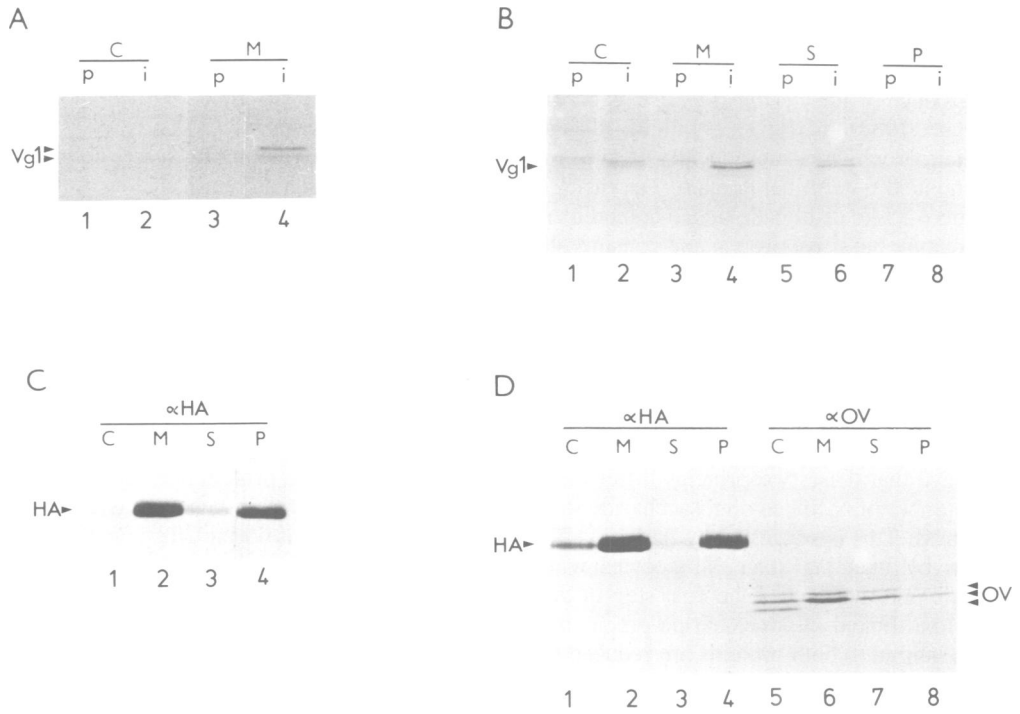

Fig. 6. Vg1 segregates as a secretory protein. (**a**) Stage VI oocytes were radiolabelled, fractionated on a 20% sucrose step and each fraction immunoprecipitated as described in Figure 1. **Tracks 1** and **2** cytosolic (C) fraction; **tracks 3** and **4** membrane fraction. (**B**) Stage VI oocytes were prepared as described in (A). Prior to immunoprecipitation membrane fractions were treated with sodium carbonate and centrifuged to generate supernatant (S) and pellet (P) fractions. (**C**) Oocyte fractions used in (B) were also immunoprecipitated with anti-haemagglutinin (HA) antibodies. These oocytes had been injected with HA mRNA prior to radiolabelling. (**D**) A second batch of oocytes was injected with both HA and ovalbumin (OV) mRNA prior to labelling and fractionation as described in (A) and (B). Fractions were then immunoprecipitated with antibodies to HA (**tracks 1–4**) and OV (**tracks 5–8**). The lower band in track 5 is unglycosylated and miscompartmented ovalbumin, the two higher bands in tracks 5–8 are glycoslyated ovalbumin. Vg1 was not immunoprecipitated from this batch of oocytes as it co-migrates with the ovalbumins.

Vg1 segregates into membranes in oocytes and embryos but is not an integral membrane protein

We have demonstrated that Vg1 is a N-glycosylated protein. Since the enzymes responsible for N-glycosylation are segregated within the lumen of the endoplasmic reticulum (ER) and Golgi apparati (Kornfeld and Kornfeld, 1985), Vg1 protein must be translocated into the lumen of the ER during its synthesis. We have shown previously that a simple sucrose gradient analysis of oocyte (and embryo) homogenates can be used to distinguish cytosolic from membrane or secretory proteins (Colman and Morser, 1979; Colman *et al.*, 1981; Krieg *et al.*, 1984). In order to confirm the segregation profile of Vg1 protein into membranes, labelled lysates from stage VI oocytes were layered onto sucrose step gradients in order to generate cytosol and membrane fractions. Each fraction was then immunoprecipitated with either preimmune or Vg1 antisera. From Figure 6A it is clear that in the stage VI oocyte Vg1 protein co-segregates with membranes. A similar result was obtained following fractionation of stage 10 embryos (data not shown).

The membrane vesicles described above contain several classes of protein, including secretory proteins as well as peripheral and integral membrane proteins. To some extent these protein classes can be distinguished by extraction of membranes with sodium carbonate at pH 11 followed by centrifugation (Fujiki *et al.*, 1982). Luminal and peripheral membrane proteins are released by this procedure whilst integral membrane proteins are retained. In order to establish the topological fate of Vg1 proteins we performed the following experiment: oocytes injected either with haemagglutinin (HA) mRNA or both HA and ovalbumin (OV) mRNAs were radiolabelled and then fractionated into cytosol and membrane fractions. Aliquots of the membrane fractions were extracted with sodium carbonate and both supernatants and washed membrane fractions collected. Samples were examined by immunoprecipitation and electrophoresis for the presence of Vg1, OV and HA; these latter two proteins serve as markers for the fate of secretory (OV) and integral membrane (HA) proteins. As we observed above, in the initial fractionation the majority of Vg1 segregated with the membranes in a similar manner to co-expressed HA (cf. tracks 1 and 2, Figure 6C with tracks 2 and 4, Figure 6B); we attribute the 'cytosolic' Vg1 to leakage resulting from homogenization. Indeed in a parallel fractionation of oocytes synthesizing OV, some glycosylated OV also leaked into the cytosol fraction (Figure 6D, track 5). However, there is no contamination of the membrane fraction with cytosolic proteins as shown by the exclusive presence of a non-glycosylated OV in the cytosolic fraction (Figure 6D tracks 5–8); we have previously reported that this species is miscompartmented within the oocyte (Colman *et al.*, 1981). When the prepared membranes were treated with carbonate, OV (Figure 6D, tracks 7 and 8) and Vg1 (Figure 6B, tracks 6 and 8) are released to similar extents whilst HA (Figure 6C, D, tracks 3 and 4) is retained. Clearly under these conditions Vg1 is behaving like a secretory rather than a membrane-anchored protein.

Discussion

The *Xenopus* localized maternal mRNA, Vg1 is a member of a family of polypeptide growth factors with homology

to TGF-β (Weeks and Melton, 1987). Other members of this family include several forms of TGF-β, the activins and inhibins, müllerian inhibitory substance (MIS), and the predicted protein product of the *decapentaplegic* gene of *Drosophila* (Massagué, 1987). TGF-β1 is synthesized as a 391 amino acid precursor which is subsequently cleaved to generate a 112 amino acid C-terminal domain (Derynck *et al.*, 1985). This forms a 25 kd disulphide-linked homodimer that constitutes the mature bioactive protein and contains all of the amino acid conservation within this family. Most other members of this family are processed in a similar fashion, the one exception being MIS in which the TGF-β homologous domain is not cleaved from the precursor (Cate *et al.*, 1986).

In this paper we have shown that the major protein products of Vg1 mRNA are two glycoproteins of M_r 43.5 and 45 kd. We believe that these two proteins differ only with respect to the number of N-linked oligosaccharide side chains that they possess. This is suggested by inhibiting N-linked glycosylation by injecting stage VI oocytes with tunicamycin, an antibiotic that blocks the first step in the biosynthesis of the lipid-linked oligosaccharide precursor. Following complete inhibition both proteins are reduced to a single band of M_r 40 kd whilst partial inhibition produces four proteins of 40, 42, 43.5 and 45 kd. Since the Vg1 cDNA sequence predicts three potential N-linked glycosylation sites (Weeks and Melton, 1987), we believe that these four proteins represent a single polypeptide wth 0, 1, 2 and 3 N-linked oligosaccharide side chains respectively. Confirmatory evidence for Vg1 glycosylation was obtained for both oocytes (Figure 4B) and embryos (data not shown) by digesting immunoprecipitated protein with the enzyme endo-H. In both cases the 45 and 43.5 kd proteins were reduced to a single protein of 40 kd by this enzyme.

During embryogenesis the relative prevalence of the 43.5 and 45 kd proteins changes. In the oocyte and early embryo all three potential glycosylation sites are utilized, but during the late blastula and early gastrula stages only two sites are used. Experiments involving translation of injected influenza haemagglutinin RNA at different stages of embryogenesis excludes global changes in the capacity of the gastrula machinery to perform N-glycosylation. In this protein all five sites were glycosylated at all stages (data not shown). One explanation for the new glycosylation pattern is that a change to the ER luminal environment of the blastula/gastrula embryo, such as the production of a new protein with affinity to Vg1, results in a conformational change in the newly synthesized Vg1 protein. This could mask one of the N-glycosylation sites; we have previously shown that mutations in chick ovalbumin can result in changes in the usage of glycosylation sites during expression in oocytes, presumably as a result of a conformational change (Tabe *et al.*, 1984). The significance of this change in the Vg1 glycosylation is not at all clear, but it is tempting to speculate that it is not trivial. Perhaps it relates to the expected processing of this protein as discussed below.

The demonstration that Vg1 is a glycoprotein indicates that it is translocated to the lumen of the ER, since the enzymes responsible for the first stages of N-linked glycosylation are segregated within the lumen of this organelle (Kornfeld and Kornfeld, 1985). By isolating membrane and cytosol fractions from oocytes and embryos we have shown that this is indeed the case. However, we were unable to detect a difference between the apparent mol. wt of the Vg1 protein made *in vitro* and the segregated product made in oocytes in the presence of tunicamycin. This might suggest that the signal sequence is not removed from Vg1 and the application of Von Heijne's rules (Von Heijne, 1983, 1984) to this sequence failed to identify a clearly defined signal cleavage site. Although signal removal does not always lead to a change in apparent mol. wt (Chiroco *et al.*, 1988) we were interested in the possibility that an uncleaved signal sequence could act as a membrane anchor. In order to define the relationship of Vg1 protein with the oocyte membranes more precisely, we have prepared oocyte membranes and washed them with high pH media, a procedure which allows integral membrane proteins to be distinguished from luminal and peripheral membrane proteins (Fujiki *et al.*, 1982). Using the distribution of the secretory protein ovalbumin and the integral membrane protein haemagglutinin as markers, we found that Vg1 protein behaved as a luminal protein. We conclude that Vg1 is not an integral membrane protein, whilst its diffusional behaviour would indicate that it is also not tightly bound to any accessory membrane proteins (see Ceriotti and Colman, 1988).

Synthesis of both Vg1 proteins is first detected in stage IV oocytes and continues throughout early embryogenesis until the late gastrula. We find that the protein cannot be detected at late stages when the message has been degraded (Rebagliati *et al.*, 1985). Since the mRNA is present throughout oogenesis at equivalent levels (Melton, 1987) it is probable that some form of translation control is in operation, although protein synthesis followed by rapid degradation in the early stages of oogenesis cannot be excluded. It is interesting that translation of this message is only apparent once its localization to the subcortical cytoplasm of the vegetal hemisphere of the developing oocyte has begun (Melton, 1987).

In both oocytes and embryos the Vg1 proteins are only synthesized in the vegetal hemisphere, as expected from the localization of Vg1 mRNA to this region (Melton, 1987; Weeks and Melton, 1987). However, in the oocyte both proteins are able to diffuse into the animal hemisphere, probably through the lumen of the ER. We have previously shown that lateral diffusion of secretory proteins (ovalbumin and the ER luminal protein, GRP78) but not membrane proteins (rotavirus VP10 and influenza haemagglutinin), occurs rapidly through the ER lumen into the animal hemisphere from localized sites of translation (Ceriotti and Colman, 1988; Drummond *et al.*, 1985). Since the 45 kd Vg1 protein appears to be stable, its accumulation in both hemispheres of the developing oocyte could have important implications for the possible role(s) of Vg1 in early embryogenesis (see below). To determine the stability of Vg1 during embryogenesis we aim to label oocyte proteins with ^{35}S and, following reimplantation into the body cavity of an adult female, obtain fertilized eggs and embryos (Holwill *et al.*, 1987) for analysis of their ^{35}S-labelled Vg1 content.

The predicted sequence of Vg1 protein contains a series of positively charged amino acids (Arg-Arg-Lys-Arg) just proximal to the TGF-β homologous domain (Weeks and Melton, 1987). Since similar sequences in other members of the TGF-β family appear to represent peptidase processing sites for the production of a mature C-terminal subunit, a similar processing event was expected of Vg1, releasing a polypeptide of 114 amino acids. So far we have been unable

to detect this polypeptide at any stage of development, in either tissue homogenates or culture medium. In view of the Vg1 fragment used to make the antigen it is unlikely that our antisera would not be able to precipitate this processed polypeptide. We therefore conclude that most (if not all) of Vg1 protein synthesized in oocytes is neither processed nor secreted and that this may represent a major level of control in Vg1 function. Although this is clearly true of the oocyte we cannot yet extend this conclusion to embryos since we are unable to impose true 'chase' conditions.

The homology between Vg1 and TGF-β suggests a possible role for the Vg1 protein product during early embryonic development. At least two members of the TGF-β family, TGF-β1 and TGF-β2, have been shown to have some mesoderm inducing activity when applied to animal poles isolated from *Xenopus* blastulae, either alone or in combination with fibroblast growth factor (FGF: Kimelman and Kirschner, 1987; Rosa *et al.*, 1988). In addition, the *Xenopus* XTC cell line produces a potent mesoderm inducing activity (Smith, 1987), that has many properties consistent with it being a member of the TGF-β family (Rosa *et al.*, 1988; Smith *et al.*, 1988). This suggests that Vg1 may be one component of the system responsible for mesoderm induction in *Xenopus* embryos (Weeks and Melton, 1987).

In amphibians mesoderm appears to arise as the result of an inductive signal(s) emanating from the vegetal hemisphere of the blastula (Nieuwkoop, 1969; Dale *et al.*, 1985; Gurdon *et al.*, 1985), a signal(s) which also confers dorso-ventral polarity to the induced mesoderm (Boterenbrood and Nieuwkoop, 1973; Gimlich and Gerhart, 1984; Dale *et al.*, 1985; Dale and Slack, 1987b). This signal would not appear to be Vg1 alone since we were unable to detect any reproducible difference in the translation of Vg1 between the dorsal and ventral halves of embryos. Another likely component is FGF which can induce ventral mesoderm (Slack *et al.*, 1987) and has a homologue in the early *Xenopus* embryo (Kimelman and Kirschner, 1987). However, it is still feasible that differences in the expected processing of Vg1 may exist between different regions of the embryo. Identification of the predicted processed product would be required to clarify this point.

An alternative role for Vg1 may be that of an endodermal determinant. We have shown that during embryogenesis the newly synthesized protein is only found in cells of the vegetal hemisphere at a time when these cells are being specified to form endoderm (Heasman *et al.*, 1984; Wylie *et al.*, 1987). Furthermore, at least one of the heterologous mesoderm inducing factors, the vegetalizing factor extracted from chick embryos (Born *et al.*, 1972), is able to induce endoderm formation in isolated amphibian animal pole tissue (Minuth and Grunz, 1980). This suggests that the primary repsonse to this factor may be to differentiate endoderm, the mesoderm arising as a result of secondary interactions between the endoderm and any remaining ectoderm. At present it is not known if such a scheme could be applied to other mesoderm inducing factors, such as XTC-MIF and TGF-β.

Materials and methods

Embryos and oocytes

Oocytes, staged according to Dumont (1972), were obtained from females of the frog *X.laevis* and maintained and where necessary microinjected as described by Colman (1984). Oocytes for the staged series were obtained following digestion of the ovary with 2 μg/ml of collagenase (Type IV, Sigma) in Ca^{2+}- and Mg^{2+}-free modified Barth saline (MBS). Stage VI oocytes were matured by the addition of 2 μg/ml of progesterone (Sigma). Oocytes were labelled by incubating in MBS plus 1 mCi/ml of [^{35}S]methionine (1415 Ci/mmol, Amersham) for 6–18 h at 18°C. Chases were performed in MBS plus 2 mM cold methionine. Oocytes were bisected into animal and vegetal halves as described by Ceriotti and Colman (1988).

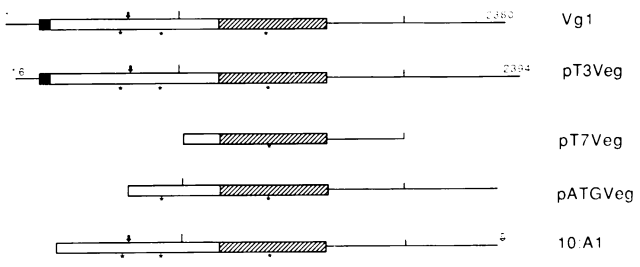

Fig. 7. Vg1 constructs. For simplicity only the Vg1 cDNA sequences of the various recombinant plasmids are displayed. **Vg1**, near full length Vg1 cDNA construct described by Weeks and Melton (1987). **pT3Veg**, a Vg1 cDNA isolated from a lambda ZAP oocyte cDNA library which includes nucleotides 16–2394 inclusive. **pT7Veg**, the Vg1 *Bgl*II–*Bgl*II fragment cloned into expression vectors to produce the T7–Vg1 and β-Gal–Vg1 fusion proteins. **pATGVeg**, the Vg1 *Nco*–*Bam*HI fragment cloned into pSPOV to produce the truncated Vg1 transcript (Vg1t). **10:A1**, the Vg1 clone isolated from λ gt10. The boxed areas of each construct represents open reading frame: solid shading, the putative signal sequence; cross-hatched shading, TGF-β homologous domain; stars, potential N-glycosylation sites; open arrow, *Bam*HI site; closed arrow, *Nco*I site; straight lines, *Bgl*II sites.

Fertilized embryos were obtained using methods described by Dale and Slack (1987a) and staged according to Nieuwkoop and Faber (1967). Embryo fragments were isolated using techniques described by Dale *et al.* (1985). All embryos and embryo fragments were cultured in full strength MBS, when required embryos were transferred to 10% (v/v) MBS to allow normal gastrulation. Embryos were labelled by injection of ~1 μCi (50 nl) of [^{35}S]methionine into early cleavage blastomeres or into the blastocoel of later stage embryos. Alternatively, embryo fragments were labelled by culturing in 100 μCi/ml of [^{35}S]methionine in MBS. All embryos and embryo fragments were cultured for 2 h.

Labelled, unfrozen oocytes and embryos were fractionated on sucrose step gradients using the 'cushion' method described by Colman (1984). The supernatants containing cytosolic components were stored at −20°C, whilst the pellets containing membranes were resuspended in either homogenization buffer (1% Triton X-100, 100 mM NaCl, 20 mM Tris–HCl, pH 7.4, 1 mM PMSF, 1 μM pepstatin) or in T buffer (50 mM NaCl, 10 mM magnesium acetate, 10 mM Tris–HCl, pH 7.4). Pellets dissolved in the latter buffer were diluted with an equal volume of 200 mM sodium carbonate (pH 11) and incubated on ice for 30 min. This was layered onto 200 μl 0.5 M sucrose in 0.1 M sodium carbonate (pH 11) and centrifuged in a Beckman TL100 at 100 000 *g* for 1 h at 4°C. The resulting supernatant was neutralized with HCl and the pellet resuspended in homogenization buffer. In some experiments oocytes were injected with chick oviduct poly(A)$^+$ RNA or synthetic RNA encoding influenza haemagglutinin (pSP HA_{WT}) prepared as described by Ceriotti and Colman (1988). The encoded proteins provide markers for the segregation of secretory proteins (ovalbumin) and integral membrane proteins (haemagglutinin).

Production of fusion proteins

Poly(A)$^+$ RNA extracted from animal and vegetal halves of stage VI oocytes (Kressman *et al.*, 1978) was used to screen differentially an oocyte λ gt10 cDNA library. A single class of vegetal specific clones were identified and one member of this class (10:A1, Figure 7) contained a 2.4 kb insert which was used for further analysis. Restriction mapping and subsequent sequencing of this clone demonstrated that it was almost identical to the Vg1 clone described by Melton and colleagues (Rebagliati *et al.*, 1985; Weeks and Melton, 1987).

A T7–Vg1 fusion protein was obtained by cloning the 1126 bp *Bgl*II (Figure 7) fragment of the Vg1 cDNA clone into the *Bam*HI site of the T7 expression vector pET-3c (Rosenberg *et al.*, 1987). The resulting vector (pT7Veg, Figure 7) contains the putative open reading frame coding for the C-terminal 156 amino acids of Vg1 protein, including all 114 amino

acids of the TGF-β homologous domain (Weeks and Melton, 1987), in frame with the N-terminal 11 amino acids of T7 *gene 10* protein and an arginine coded for by the linker sequence. This was confirmed by sequencing the insert and surrounding vector DNA, and the construct was expected to yield a fusion protein of M_r 19.7 kd. Cloning was carried out in the *Escherichia coli* strain DH1. The fusion vector was reisolated and then transformed into the *E.coli* lysogen BL21 (DE3) which contains a single copy of the gene for T7 RNA polymerase under the control of the inducible *lac*UV5 promoter (Studier and Moffatt, 1986). Cells were cultured until an OD reading of 0.8 (600 nm) was reached and then induced by the addition of 0.4 mM IPTG and cultured for a further 3 h. The major protein synthesized by these cells had an M_r of 20 kd and was absent from uninduced cells as well as induced cells transformed with a T7−Vg1 fusion vector containing Vg1 sequences in reverse orientation. From this we deduced that the major 20 kd protein was the T7−Vg1 fusion protein and it was used to generate polyclonal antibodies.

A second fusion protein was produced by cloning the same *Bgl*II fragment of the Vg1 cDNA clone (see above) into the *Bgl*II site of the pEX2 expression vector (Stanley and Luzio, 1984), to generate a *cro−lacZ*−Vg1 fusion. Following transformation into the *E.coli* strain mc1061-CIts, the β-Gal−Vg1 fusion protein was induced by transferring cells from 28 to 42°C at early log phase. This fusion protein was used to check the specificity of antibodies raised against the T7−Vg1 fusion protein.

Production of polyclonal antibody

Induced cells were spun down and resuspended in 50 mM Tris−HCl (pH 7.9), 1 mM EDTA, 100 mM NaCl, 0.25 mg/ml lysozyme and incubated at room temperature for 15 min. Triton X-100 was added to 0.2% (v/v), $MgCl_2$ to 10 mM and DNase I (Sigma) to 1 μg/ml. After incubating for 30 min at room temperature the sample was centrifuged at 10 000 r.p.m. for 10 min at 4°C and the supernatant discarded. The fusion protein was separated on 15% preparative gels by SDS−PAGE (Laemmli, 1970) and eluted from the acrylamide in 50 mM Tris−HCl, pH 7.9, 0.1 mM EDTA, 1 mM DTT, 0.1 M NaCl, 0.1% SDS for 4 h at 30°C. Eluted fusion protein was concentrated 10-fold in an Amicon concentrator, mixed 50:50 with complete Freund's adjuvant and injected into the thigh of two New Zealand white rabbits. A third rabbit was injected with fusion protein still in acrylamide mixed 50:50 with complete Freund's adjuvant. Rabbits were boosted 3× with a 50:50 mixture of concentrated fusion protein and incomplete Freund's adjuvant, and then bled on a 4-weekly schedule.

RNA preparation

Chick oviduct poly(A)$^+$ RNA and a synthetic RNA (pSPHAwt RNA) encoding influenza haemagglutinin were obtained as described by Ceriotti and Colman (1988). Full length and truncated Vg1 RNAs were synthesized *in vitro* from the plasmids pT3 Veg and pATG Veg respectively, using T3 RNA polymerase (Pharmacia; pT3 Veg) or SP6 RNA polymerase (Boehringer; pATG Veg) as previously described (Ceriotti and Colman, 1988). pT3 Veg (Figure 7) was selected from a *lamda* Zap (Stratagene) oocyte cDNA library prepared by John Shuttleworth in our laboratory. pATG Veg (Figure 7) was prepared by first subcloning a *Hin*dIII fragment of a full length chicken ovalbumin cDNA from pTK2OV (Krieg *et al.*, 1984) into pSP64 (Krieg and Melton, 1987) to produce pSPOV. Excision of this plasmid with *Nco*I and *Bam*HI and replacement of the ovalbumin fragment with the *Nco*I−*Bam*HI Vg1 fragment from 10.A1 (Figure 7) results in a clone encoding a truncated Vg1 protein beginning with the N-terminal methionine from ovalbumin.

Immunoprecipitation

^{35}S-Labelled specimens were homogenized in 20 μl/oocyte (or embryo) of homogenization buffer. Samples were clarified in a microfuge at 13 000 r.p.m. for 10 min at 4°C and the supernatant transferred to a fresh tube and diluted in 5 vols of detergent buffer (100 mM Tris−HCl, pH 7.4, 1% Triton X-100, 0.5% SDS, 5 mM $MgCl_2$, 100 mM KCl, 1% Na-deoxycholate). They were then reclarified in a Beckman TL100 ultracentrifuge at 100 000 r.p.m. for 15 min at 4°C. Samples were then incubated for 1.5 h with 1 μl/oocyte (embryo) of preimmune serum, followed by a 1.5 h incubation with 10 μl/oocyte (embryo) of protein A−Sepharose (Pharmacia 20% v/v in 100 mM NaCl, 50 mM Tris−HCl, pH 7.4, 5 mM EDTA) all at 4°C. The samples were then spun at 13 000 r.p.m. and the supernatants divided into two equal sized aliquots. A concentration of 1 μl/oocyte (embryo) of either preimmune or anti-Vg1 serum was added to each tube and incubated overnight at 4°C. They were then incubated with protein A−Sepharose for 3 h at 4°C, pelleted and washed three times with detergent buffer. Finally they were resuspended in sample buffer and processed for SDS−PAGE. After electrophoresis, gels were fixed and prepared for fluorography as described by Bonner and Laskey (1974). Alternatively they were prepared with En^{3}hance (NEN) according to the manufacturer's instructions.

Oligonucleotide depletion of Vg1 mRNA

Antisense oligonucleotides against histone H4 (H4-1) and Vg1 (Veg-1 and Veg-2) were as described by Shuttleworth and Colman (1988) and Shuttleworth *et al.* (1988). Aliquots (50 nl) containing oligonucleotides (3 mg/ml) were injected into the cytoplasm of stage VI oocytes as described by Colman (1984). Oocytes were incubated overnight prior to labelling with [^{35}S]methionine for 8 h followed by immunoprecipitation of labelled extracts. For the *in vitro* study, 5 μg of *Xenopus* ovary poly(A)$^+$ RNA was annealed to 0.25 μg of either Veg-1 or H4-1 oligonucleotide in a final volume of 10 μl of 100 mM KCl, 20 mM HEPES, pH 7.1 mM EDTA, and incubated at 60°C for 20 min. Since the wheat germ contains an endogenous RNase H-like activity (Minshull and Hunt, 1986), the RNA was translated directly by the addition of 9 vols of wheat germ translation mix. Alternatively, hybrids were digested by the addition of 5 μl RNase H (25 μl/ml in 100 mM KCl, 3 mM DTT, 150 μg/ml BSA, 11.5 mM $MgCl_2$, 20 mM Tris, pH 7.5) and incubated for 30 min at 37°C (Minshull and Hunt, 1986). The depleted RNA was translated by the addition of 9 vols of translation mix.

Analysis of N-linked glycosylation

N-glycosylation of newly synthesized stage VI oocyte proteins was inhibited by injection of 40 μg/ml of tunicamycin (Sigma) as described by Colman (1984). Injected oocytes were labelled immediately for 18 h or incubated overnight in 2 μg/ml tunicamycin in MBS prior to labelling for 6 h with [^{35}S]methionine. Additionally, N-linked oligosaccharides were enzymatically removed from Vg1 protein using the enzyme endo-H (EC 3.2.1.96, ICN), as described by Colman *et al.* (1985).

Acknowledgements

We would like to thank Drs F.W.Studier and David Ish-Horowicz for providing us with the T7 vectors and stocks, Dr John Shuttleworth for reading the manuscript and providing Vg1 sequence data, Dr Alistair Strain for providing porcine TGF-β1, Dr Jane Gould for help with the photography and Dr Liz Jones for help wth rabbit immunization. This work was supported by a grant to A.C. from the Medical Research Council (UK).

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Received on December 6, 1988; revised on January 23, 1989