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Original Article

Amphibian *Bufo arenarum* vitronectin-like protein: its localization during oogenesis

MANUEL J. AYBAR, SUSANA B. GENTA, EVELINA I. VILLECCO, ALICIA N. SÁNCHEZ RIERA and SARA S. SÁNCHEZ*

Departamento de Biología del Desarrollo, Instituto Superior de Investigaciones Biológicas (INSIBIO), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Universidad Nacional de Tucumán (UNT), San Miguel de Tucumán, Tucumán, Argentina

ABSTRACT In the present study, we analyzed the localization of vitronectin-like protein in oocytes during oogenesis as well as in the serum and liver tissue of the amphibian *Bufo arenarum*. Vitronectin-like protein was purified from serum by heparin-affinity chromatography and showed to have the two biological properties in common with most animal vitronectins (VN): heparin binding activity and an RGD-dependent cell-spreading activity. SDS-PAGE of vitronectin-like protein revealed that it consists of two bands of 64 kDa and 72 kDa, while immunoblotting analyses showed that this protein strongly cross-reacts with two monoclonal antibodies against human VN. No immunofluorescent staining of vitronectin-like protein was observed in previtellogenic oocytes (stages I and II). In vitellogenic oocytes (stages III, IV and V) fluorescence was observed in the cortical cytoplasm localized in yolk platelets, extending concomitantly with the vitellogenic process. When we examined the yolk platelet formation pathway by immunoelectron microscopy, gold particles indicated that vitronectin-like protein was located on the yolk platelet precursors: multivesicular bodies and primordial yolk platelets. Gold particles also were seen sparsely distributed in all oocyte investing layers. The mean serum vitronectin-like protein concentration in amphibian animals was 127.8±11.6 μg/ml in adult males and 181.5±14.3 μg/ml in adult females. Serum vitronectin-like protein of males and females was susceptible to hormonal stimulation (17-ß estradiol). These results suggest that vitronectin-like protein is stored in the yolk platelets and may be involved in the later events of amphibian development.

KEY WORDS: *vitronectin-like protein, oogenesis, yolk platelets, extracellular matrix protein, amphibian*

Introduction

Vitronectin (VN) is a multifunctional cell-adhesive glycoprotein present in animal blood and extracellular matrix (for reviews see Preissner, 1991; Tomasini and Mosher, 1991). VN promotes spreading of cells through the Arg-Gly-Asp (RGD) sequence located near the NH_2-terminus of the molecule (Suzuki *et al.*, 1984). The cell-spreading activity is competitively inhibited by RGD-containing peptides (Yamada and Kennedy, 1987). VN is identical to the S-protein of the complement system, and also appears to have regulatory functions in the coagulation and fibrinolytic system (for reviews see Tomasini and Mosher, 1990; Preissner, 1991). It has a heparin-binding site toward the COOH-terminus, which is cryptic in native form but exposed after treatment with 8 M urea (Barnes *et al.*, 1985; Hayashi *et al.*, 1985). Based on this property, vitronectin is easily purified from human plasma by heparin affinity chromatography in the presence of 8 M urea (Yatohgo *et al.*, 1988). The procedure succeeded in purifying vitronectins from plasma or sera of many animal species using the method above. Their biological and chemical features were compared by Kitagaki-Ogawa *et al.* (1990). They showed two major biological properties in common: heparin binding activity in the presence of 8 M urea and cell-spreading activity on BHK cells (Miyazaki *et al.*, 1992). The molecular weights for these vitronectins ranged from 59-78 kDa and they showed a similar amino-terminal sequence (Kitagaki-Ogawa *et al.*, 1990). Most animal vitronectins tested cross-reacted with monoclonal antibody raised against human VN. These results indicate that the amino-terminal 5 kDa portion of animal vitronectins have well-preserved structures which are immunologically related (Kitagaki-Ogawa *et al.*, 1990). The high degree of preservation throughout evolution suggests that vitronectins are of vital importance in several cell functions

Abbreviations used in this paper: VN, vitronectin; RGD, Arg-Gly-Asp; FN, fibronectin; GRGDSP, Gly-Arg-Gly-Asp-Ser-Pro; GRGESP, Gly-Arg-Gly-Glu-Ser-Pro; MVB, multivesicular body.

***Address for reprints:** Departamento de Biología del Desarrollo, INSIBIO (CONICET-UNT), Chacabuco 461, 4000-San Miguel de Tucumán, Argentina. FAX: 54-81-248025. e-mail: mjaybar@isibio.edu.ar

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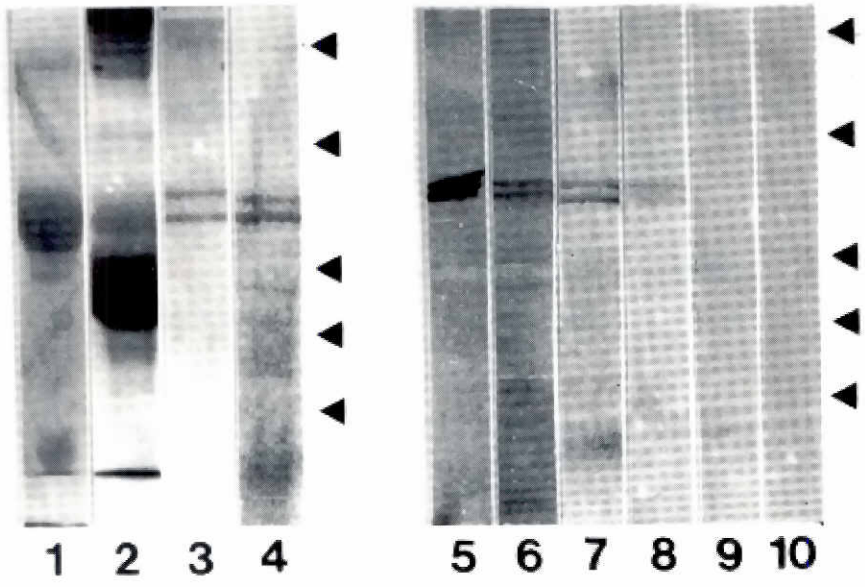

Fig. 1. SDS-PAGE and Western immunoblotting of *Bufo arenarum* serum and isolated vitronectin-like protein. (Lanes 1-4) *Stained with Coomasie Blue.* **(Lanes 5-10)** *Western immunoblots with anti-human VN antibodies. Lane 1, human blood serum (30 μg) ; lane 2, Bufo arenarum blood serum (50 μg); lane 3, purified human VN (6μg) and lane 4, purified Bufo arenarum vitronectin-like protein (6 μg). Lanes 5-6, human and Bufo arenarum blood serum (65 μg, each) stained with anti human VN antibody (Clone VIT-2); lane 7, purified Bufo arenarum vitronectin-like protein (0.5 μg) stained with anti-human VN antibody (Clone-VIT-2); lane 8, purified Bufo arenarum vitronectin-like protein (0.5 μg) stained with another anti-human VN antibody (Clone VN5-3). Lanes 9-10, 64-72 kDa bands were not stained (0.5 μg, each) with either antibody preadsorbed with an excess amount of pure human VN. On the right, molecular mass markers (from top to bottom, 205, 125, 51, 41 and 35 kDa).*

(Preissner, 1991), and are also present in various tissues (Seiffert *et al.*, 1993).

However, the role, if any, of VN in animal development and even the existence of VN in amphibian oocytes has not been established yet. Many other cell adhesion proteins such as fibronectin (Boucaut *et al.*, 1985), laminin (Darribere *et al.*, 1986), cadherin (Takeichi, 1988) and tenascin (Riou *et al.*, 1990) are known to play a role in morphogenesis during early amphibian development. Several lines of evidence suggest that these adhesion proteins and their cell surface receptors may be involved in several processes essential for cellular function during normal development (Thiery *et al.*, 1985; Darribere *et al.*, 1988; Sánchez *et al.*, 1988; Boucaut *et al.*, 1991; Johnson *et al.*, 1992). These glycoproteins were found during oogenesis and described spatially and temporally in close relationship with important developmental events (Darribere *et al.*, 1984; Riou *et al.*, 1987). Although knowledge of the structure and properties of vitronectins from mammalian and avian blood has increased, nothing is known about the expression of vitronectin during oogenesis. The presence of vitronectin in *Xenopus* serum is mentioned by Nakashima *et al.* (1992), but the localization of the corresponding protein in the oocyte and the role, if any, of vitronectin in amphibian oocytes has not been established yet.

Although limited information is available with regard to the site of VN biosynthesis, a number of cultured cells have been shown to synthesize and secrete VN (Yasumitsu *et al.*, 1993). In a murine model system Seiffert *et al.* (1991) reported that the liver is the primary site of VN biosynthesis *in vivo* (Seiffert *et al.*, 1991). Solem *et al.* (1991) also reported the presence of VN in mouse liver. Only few observations suggest the possibility that VN biosynthesis may be regulated in some situations both *in vivo* and *in vitro*, but limited information is available on the conditions or agents involved in *in vivo* regulation (Seiffert *et al.*, 1994).

The present study deals with the isolation and identification of VN-related proteins from the serum of the amphibian *Bufo arenarum* and its localization in oocytes and liver tissue. Using biochemical, immunological and immunocytochemical methods, we analyzed the distribution of vitronectin-like protein during oogenesis. As *Bufo arenarum* has a reproductive annual sexual cycle we also examined the possibility that the synthesis of VN *in vivo* might be hormonally regulated.

Results

Amphibian vitronectin-like protein was detected in the serum of adult *Bufo arenarum* toad by immunoblotting as two bands using anti-human vitronectin antibody (Fig. 1, lane 6). Both human VN and amphibian serum vitronectin-like protein were extracted and purified to homogeneity through heparin-Sepharose affinity chromatography according to Yatohgo *et al.* (1988).

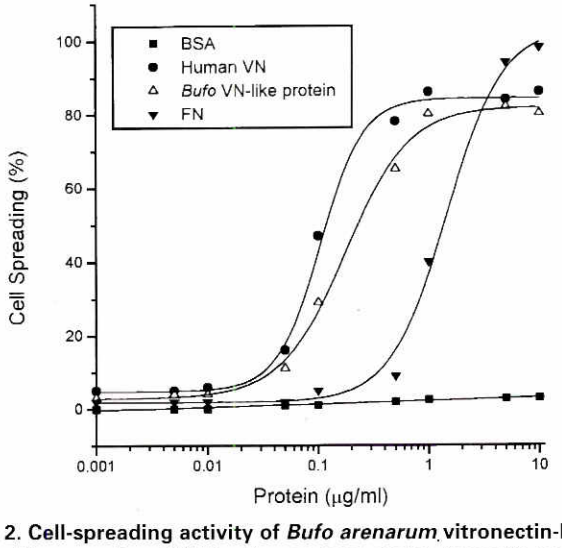

Fig. 2. Cell-spreading activity of *Bufo arenarum* vitronectin-like protein. *The spreading of BHK cells was measured on the wells coated with each protein, as described in Materials and Methods. Bufo arenarum vitronectin-like protein, human VN, FN, or BSA were allowed to coat the wells at different concentrations at 37°C for 1 h. BHK cells were added into the wells and incubated at 37°C for 90 min. The percent of cells spread was counted under a microscope.*

TABLE 1

INHIBITION OF VITRONECTIN-PROMOTED BHK CELL-SPREADING BY ANTIBODIES AND SYNTHETIC PEPTIDES

Inhibitor		Inhibition (%)	
		Human VN	*Bufo arenarum* vitronectin-like protein
None		0	0
Anti human-VN (clone VIT-2)	0.1 mg/ml	0	0
	0.2 mg/ml	0	0
	0.5 mg/ml	0	0
Anti human-VN (clone VN5-3)	0.1 mg/ml	0	0
	0.2 mg/ml	0	0
	0.5 mg/ml	0	0
GRGDSP	2 µg/ml	46	53
	5 µg/ml	75	79
	10 µg/ml	98	99
GRGESP	2 µg/ml	2	2
	5 µg/ml	3	2
	10 µg/ml	5	4

Polystyrene tissue culture dishes were coated with human VN and vitronectin-like protein (1 µg/ml) for 1 h at 37°C. To test for inhibition for antibodies, the plates were rinsed and incubated with antibodies against human VN for 1 h. The plates were rinsed and incubated with $1x10^4$ BHK cells in 0.1 ml adhesion medium. Inhibition by synthetic peptides was tested by incubating human VN and VN-like protein-coated plates with BHK cells and peptides simultaneously.

Basically the same elution patterns were obtained for human and amphibian sera. VN was recovered with an efficiency of 20-30% for human and of 10-15% for *Bufo arenarum*. The yield of *Bufo* vitronectin-like protein was approximately 1-2 mg from 100 ml of amphibian serum.

SDS-PAGE of heparin-affinity isolated vitronectins revealed slight differences in molecular weight between human and *Bufo arenarum*. Two bands of 65-75 kDa and 64-72 kDa were observed for human and *Bufo*, respectively (Fig. 1, lanes 3 and 4). Amphibian vitronectin-like protein strongly reacted with two anti-human VN antibodies (Fig. 1, lanes 7, 8, respectively). These bands were not stained with either of the two antibodies used (Clone VIT-2 Sigma Chemical Co. and Clone VN5-3, Panvera Corp.), which had been previously preadsorbed with human VN (Fig. 1, lanes 9, 10), nor with mouse non-immune serum (data not shown). Thus, these results show that *Bufo arenarum* vitronectin-like protein is immunologically cross-reactive with anti human VN antibodies.

We studied whether *Bufo* vitronectin-like protein had a cell-spreading activity as human VN did. Purified *Bufo* vitronectin-like protein was assayed for spreading of BHK fibroblastic cells as shown in Figure 2. *Bufo* vitronectin-like protein was efficient in mediating spreading of BHK cells in a dose-dependent manner. *Bufo* vitronectin-like protein and human VN induced a similar extent of cell-spreading. The dose response curve of the cell-spreading activity showed a half-maximal concentration of 0.1 µg/ml, a result similar to that induced by human VN and 9-10-fold higher than the specific activity induced by FN (Fig. 2).

RGD-containing peptides are known to compete with VN for binding to VN receptors. These peptides inhibit RGD-dependent cell-spreading when added exogenously. Accordingly, we examined the effect of RGD-peptides on *Bufo* vitronectin-like protein-mediated cell-spreading. GRGDSP peptide inhibited the cell-spreading activity of *Bufo* vitronectin-like protein and human VN in a dose-dependent manner (Table 1). In contrast with the above, a control GRGESP peptide did not significantly inhibit the cell-spreading activity of the *Bufo* vitronectin-like protein. The effect of RGD peptide suggests the presence of a cell-spreading active RGD-sequence within *Bufo* vitronectin-like protein in common with animal vitronectins.

In order to test whether the two monoclonal antibodies used in this study (Clone VIT-2, Sigma Chemical Co. and Clone VN5-3, Panvera Corp.) were able to block adhesion to amphibian serum vitronectin-like protein, the BHK cell spreading activity was assayed by incubating *Bufo* vitronectin-like protein coated plates (1 µg/ml) with anti human VN antibodies for 1 h, rinsing and incubating them with BHK cells. Neither anti-human VN antibody was able to inhibit the cell-spreading activity of *Bufo arenarum* vitronectin-like protein (Table 1).

The morphology of the spread BHK cells on the *Bufo* vitronectin-like protein was indistinguishable from that on human VN (Fig. 3A,B). The shape of the cells on vitronectin-like protein was slightly different from that of the ones on FN (Fig. 3C), in which the BHK cells were more extended.

The distribution of vitronectin-like proteins in oocytes and in liver tissue was then investigated by immunofluorescence on sections using two anti-human vitronectin antibodies (Clone VIT-2, Sigma Chemical Co. and Clone VN5-3, Panvera Corp.). To demonstrate that the immunofluorescence was not due to non-specific binding of the antibody, two different controls were performed. In the first, we treated ovarian follicles and liver sections with the antibody preadsorbed with human VN in a antigen/antibody ratio of 2:1 (dilution 1:120). No specific fluorescence was detected when those sections were revealed by the labeled secondary anti-mouse FITC-labeled antibody, except for a weak autofluorescence from the vitelline envelope. In the second control, we observed no labeling when the secondary antibody was used alone.

Localization of vitronectin-like proteins in Bufo arenarum oogenesis

Vitronectin-like proteins were observed throughout the course of oocyte development. Their intracellular localization, dependent on the oocyte stage, was especially considered. Similar distribution pattern of vitronectin-like protein was obtained with the two anti-human vitronectin antibodies used in this study.

Figure 4 shows the results obtained using an anti-human vitronectin antibody (clone VIT-2). In previtellogenic oocytes (stages I, II and III, Fig. 4A) the occurrence of vitronectin-like proteins was not seen in the cytoplasm. Only a slight fluorescence was observed in the surrounding layers of the oocytes, corresponding to the follicular layer and/or to the growing vitelline envelope. The ovarian blood vessels showed a strong luminal fluorescence suggesting a substantial presence of this protein at this location.

During the course of vitellogenesis (stage IV to V) the localization of vitronectin-like protein differed from that previously described in that this was mainly found in cytoplasm. At stage IV, preferential fluorescence was observed at the periphery of the oocyte localized in the yolk platelets, whereas the yolk-free cytoplasm showed less labeling (Fig. 4C). The stained area extended to subcortical cytoplasm at this stage. The stained organelles were small yolk platelets and nascent forms of large yolk platelets.

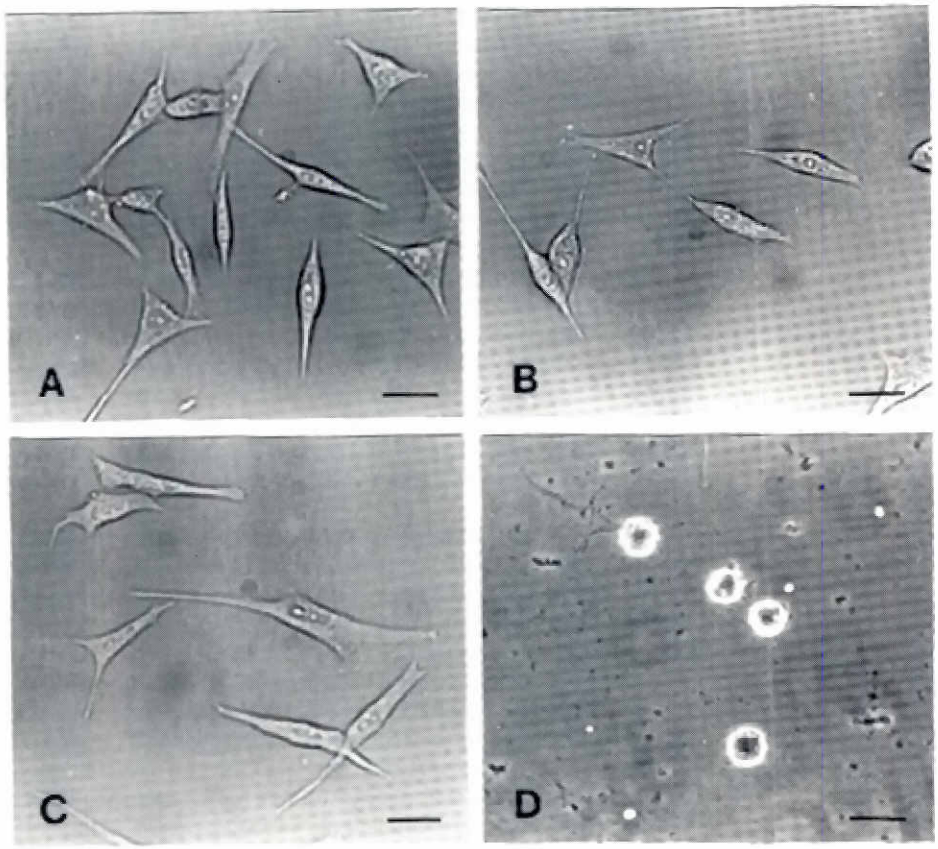

Fig. 3. Morphology of BHK cells. *Spreading of BHK cells was induced as described in the previous legend.* **(A)** *Vitronectin-like protein.* **(B)** *Human VN.* **(C)** *FN.* **(D)** *BSA. Each protein was coated at a concentration of 1 μg/ml. Bars, 10 μm.*

In oocytes at stage V (Fig. 4E), the width of the fluorescent stained-cytoplasm increased and occupied nearly the whole oocyte cytoplasm concomitant with the vitellogenic process.

Full-grown oocytes (stage VI, Fig. 4G) showed a uniform labeling but the staining decreased when compared with the earlier stages. Apparently, the labeling in the large yolk platelets was less intense than in the yolk platelets nascent form.

During vitellogenic stages (stages IV-VI) we observed a slight staining in the germinal vesicle. No fluorescence was ever detected when previtellogenic oocyte sections (Fig. 4B) were incubated with anti-human VN antibody preadsorbed with human VN. Moreover, control sections of vitellogenic oocytes did not show fluorescence when treated with VN-preadsorbed anti-human VN antibody (Fig. 4I).

Immunoelectron microscopic localization of vitronectin-like protein in Bufo arenarum yolk platelets

During vitellogenesis, the mode of yolk deposition involves well-established cytoplasmic structural features: a cortical tubular membrane system, multivesicular bodies, an organelle-to-organelle fusion process during yolk platelet formation, and finally a deposition of yolk constituents in a paracrystalline array (for a further detailed description, see Bauer and Richter, 1990; Richter and Bauer, 1990). In order to determine the occurrence of *Bufo* vitronectin-like protein in the multivesicular bodies (MVB) and yolk precursors, electron microscope immunocytochemistry using two monoclonal antibodies was used. Similar distribution pattern of vitronectin-like protein was obtained with the two anti-human vitronectin antibodies used in this study.

Figure 5A shows the content of an MVB located in the stage IV-V oocyte cortex. The condensed yolk-containing endosomes and primordial crystalline yolk platelets could be seen labeled by gold particles. Gold particles were also seen on numerous endosomes closely related to primordial yolk platelets undergoing yolk crystallization and releasing their content into this yolk precursor (Fig. 5B). In addition, gold particles were also deposited inside the MVB either near two fusing condensed primordial yolk platelets (Fig. 5C). No gold label was detected in the cytoplasm or in any other organelle of the oocyte.

No staining was observed when sections were treated with human VN preadsorbed anti-human VN antibody (Fig. 5D) or with non-immune serum (data not shown).

Immunocytochemical and immunoelectron microscope detection of vitronectin-like protein in the ovarian oocyte envelopes

Figure 6 (A,B and C) shows labeling patterns of the immunoelectron microscope analysis of the oocyte investing envelopes (stages IV-V): theca, follicle cells and vitelline envelope.

The gold particles were observed scarcely labeling the theca layer (Fig. 6A). In follicle cells gold particles were present on the cytoplasm, but neither mitochondria nor other structures were labeled (Fig. 6B). When we analyzed this layer by indirect immunofluorescent microscopy, follicle cells showed an intense fluorescence (data not shown).

As shown in Figure 6C, the vitelline envelope has two different zones, the outer margin with tightly packed fibrous bundles (follicle cell surface) and the inner margin (oocyte surface) that appeared less uniform showing microvilliar processes emanating from the egg surface and macrovilliar processes extending from the follicular cells. Pores leading into tunnels were seen where the macrovilliar processes permeated the envelope.

The gold particles were found mainly in the inner and outer oocyte vitelline envelope. Tunnels were poorly immunoreactive. The oocyte cytoplasm also showed few gold particles. Figure 6D shows a control section with no gold label on the surrounding oocyte envelopes when treated with human VN- preadsorbed anti-human VN antibody.

Immunohistochemical detection of vitronectin-like protein in amphibian liver

Distribution of vitronectin-like protein in liver tissue was examined by indirect immunofluorescence. Using the anti-human VN

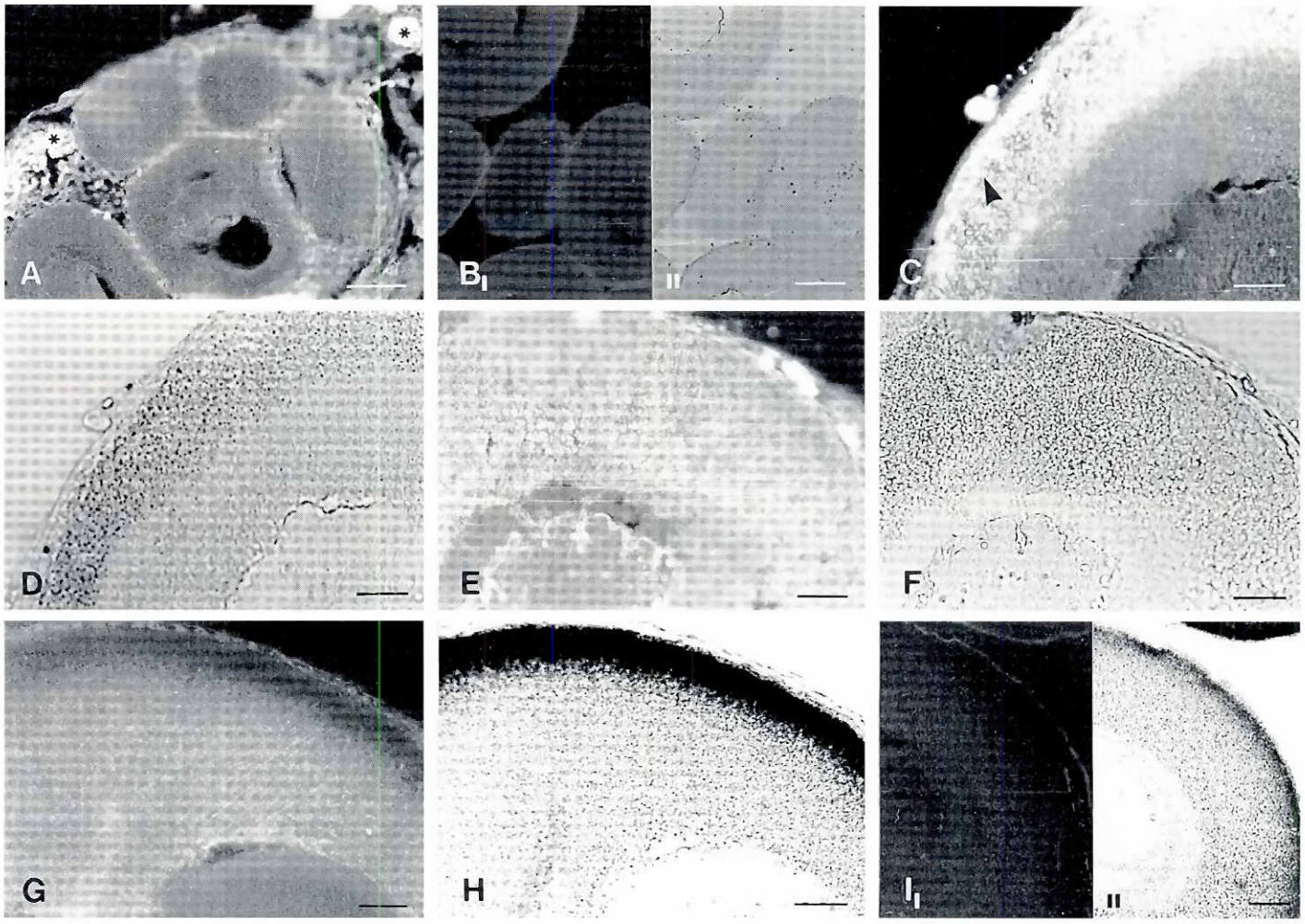

Fig. 4. Immunolocalization of vitronectin-like protein during *Bufo arenarum* oogenesis. (A, B-I, C, E, G, I-I) *Immunofluorescence.* **(B-II, D, F, H, I-II)** *Corresponding bright field. Immunofluorescent micrographs of ovarian sections treated with anti-human vitronectin mouse monoclonal antibody (A,C,E,G). Controls were performed with anti-human vitronectin antibody preadsorbed with an excess amount of human vitronectin (B,I), and then with the FITC-labeled antibodies.* **(A)** *Previtellogenic oocytes (stage I, II, and III) are not stained. Note the ovarian blood vessels strongly labeled (asterisks).* **(B-I)** *Control section of previtellogenic oocytes showing no fluorescent staining.* **(C)** *Vitellogenic oocyte (stage IV) showing fluorescence at the cortical cytoplasm. Arrowhead shows staining of small yolk platelets and nascent forms of large yolk platelets.* **(D)** *Stage IV oocyte.* **(E)** *Stage V oocyte shows fluorescence mostly occupying the whole cytoplasm.* **(F)** *Stage V oocyte.* **(G)** *Full-grown oocyte (stage VI) shows a uniform and less intense labeling.* **(H)** *Stage VI oocyte, the excentrical position of nucleus indicates the animal part of the oocyte.* **(I)** *Control section shows non-staining of vitellogenic oocyte (stage V). Bars: A,B, 50 μm; C-I, 100 μm.*

mouse monoclonal antibody, labeling of liver sections revealed that sheets of hepatocytes, sinusoids, portal and central veins were intensely positive.

Figure 7A shows a portal tract with intense labeling in portal vein content and in its endothelial wall (Fig. 7B), whereas bile ductus, hepatic arterial branch and lymphatic vessel appeared less fluorescent.

In order to examine the possibility that the vitronectin-like protein detected in hepatocytes by immunofluorescence might be plasma derived rather than produced by the positive staining hepatocytes, we exhaustively perfused livers. After a 3 h perfusion treatment, the sheets of hepatocytes were still strongly labeled, but wall vessels had lost their immunoreactivity (Fig. 7C). No fluorescent staining was seen in sections treated with anti-human vitronectin antibodies previously adsorbed with human vitronectin (Fig. 7D).

Quantitation of vitronectin-like protein in amphibian serum

Vitronectin-like protein was found in normal male and female amphibian serum detected by an ELISA method (see Materials and Methods). There was no significant difference between vitronectin-like protein concentration measured in serum and plasma (EDTA). The mean concentration of vitronectin-like protein in serum from amphibian adults was 181.5±14.3 μg/ml for females and 127.8±11.6 μg/ml for males.

Amphibians, like other oviparous vertebrates, accumulate large quantities of yolk proteins within their oocytes during the vitellogenic phase of the hormonal regulated reproductive cycle. The developing oocytes are highly specialized for the specific accumulation of extraovarian yolk protein precursors. The bulk of yolk proteins is mainly produced by the liver under the control of sexual hormones. The stimulation of yolk protein synthesis by estrogen is character-

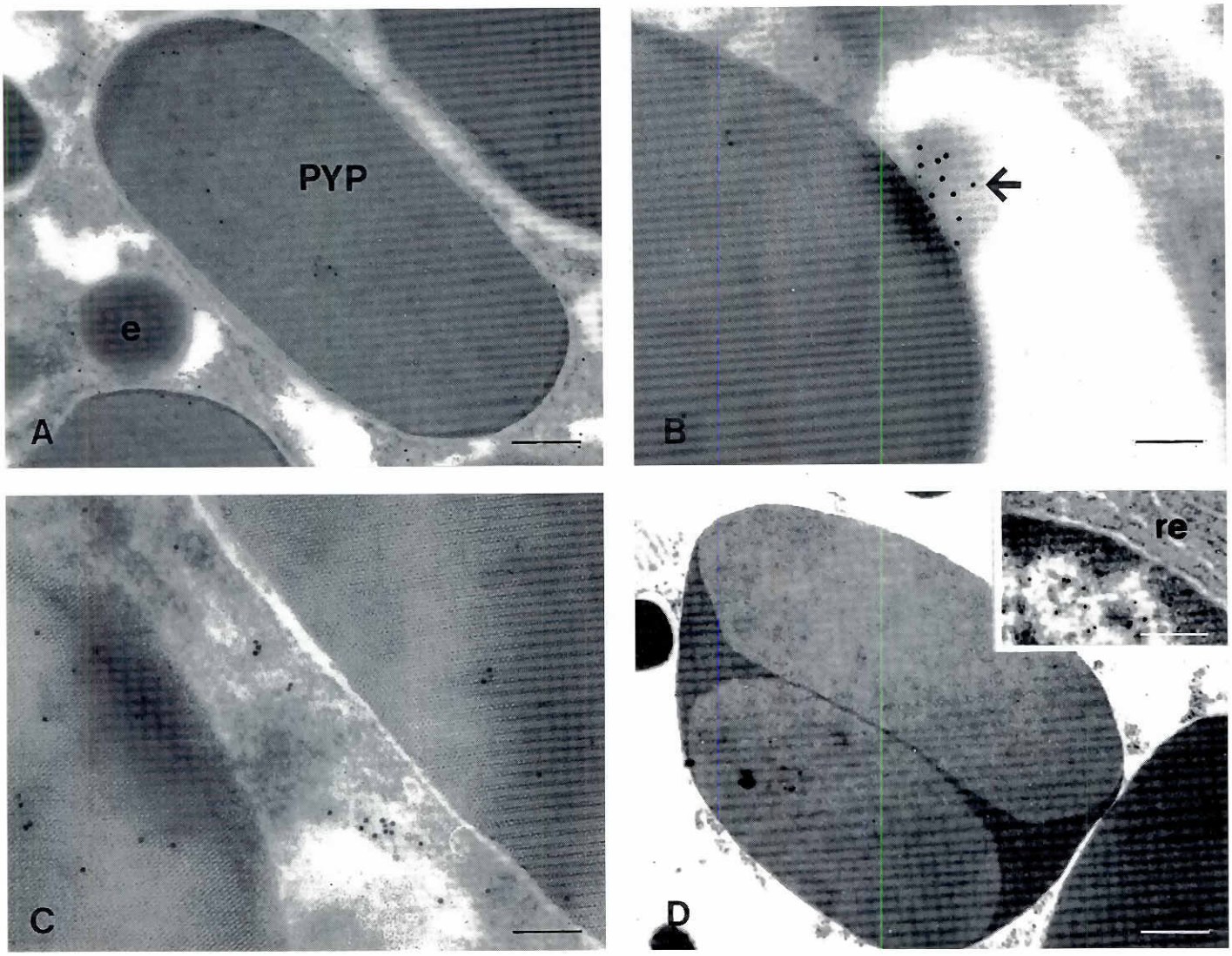

Fig. 5. Immunoelectron microscopy of vitellogenic oocytes. *Ultrathin sections of stage IV-V oocytes treated with anti-human vitronectin mouse monoclonal antibody* **(A, B, C)**. *Control section performed incubating the anti-human vitronectin antibody with an excess amount of human vitronectin* **(D)**. **(D-inset)** *shows the intensity and location of an established marker (anti-histone monoclonal mouse antibody, clone 1B3, Boehringer, Germany). Gold particles were observed on the nucleus, while no immunoreactivity was detected in the cytoplasm or in any other organelle; re: endoplasmic reticulum.* **(A)** *shows the content of an MVB located in the stage IV-V oocyte cortex. The condensed yolk-containing endosomes (e) and primordial crystalline yolk platelets (PYP) could be seen labeled by gold particles.* **(B)** *Gold particles were also seen on numerous endosomes closely related to primordial yolk platelets undergoing yolk crystallization (arrow).* **(C)** *Gold particles were also deposited inside the MVB either near two fusing condensed primordial yolk platelets. No gold label was detected in the cytoplasm or in any other organelle of the oocyte.* **(D, inset)** *Control sections. Bars: A, 0.2 μm; B,C, 0.1 μm; D, 0.2 μm, inset, 0.15 μm.*

istic of female non-mammalian vertebrates. On the basis of this fact, we examined the possibility that the synthesis of vitronectin-like protein might be hormonally regulated. We also analyzed the *in vivo* effects of estrogen on serum vitronectin-like protein levels in adult toads.

The dose chosen for injection, based on our experience with estrogen effects in this amphibian, was 100 μg/100 g body weight (Sánchez and Sánchez Riera, 1977), in accordance with doses typically used in non-mammalian species (birds, fish, *Xenopus*) for such studies. For review of estrogen doses and actions in vertebrates see Callard and Klots (1973).

As shown in Table 2, estrogen treatment produced an increase in the serum vitronectin-like protein levels. An increased immunostaining of hepatocytes was seen (data not shown) after 24 h of estrogen injection. Estrogenized males responded in the same way to hormonal treatment. These results strongly suggest that the vitronectin-like protein was synthesized in the liver and secreted therefrom into circulation under the influence of estrogen.

Discussion

Although VN was initially isolated and described in humans (Barnes and Silnutzer, 1983; Hayman *et al.*, 1983), recent studies provide evidence that VN is also present in various animal species. Vitronectins from different vertebrates and invertebrates have similar features, except for some variations in apparent molecular

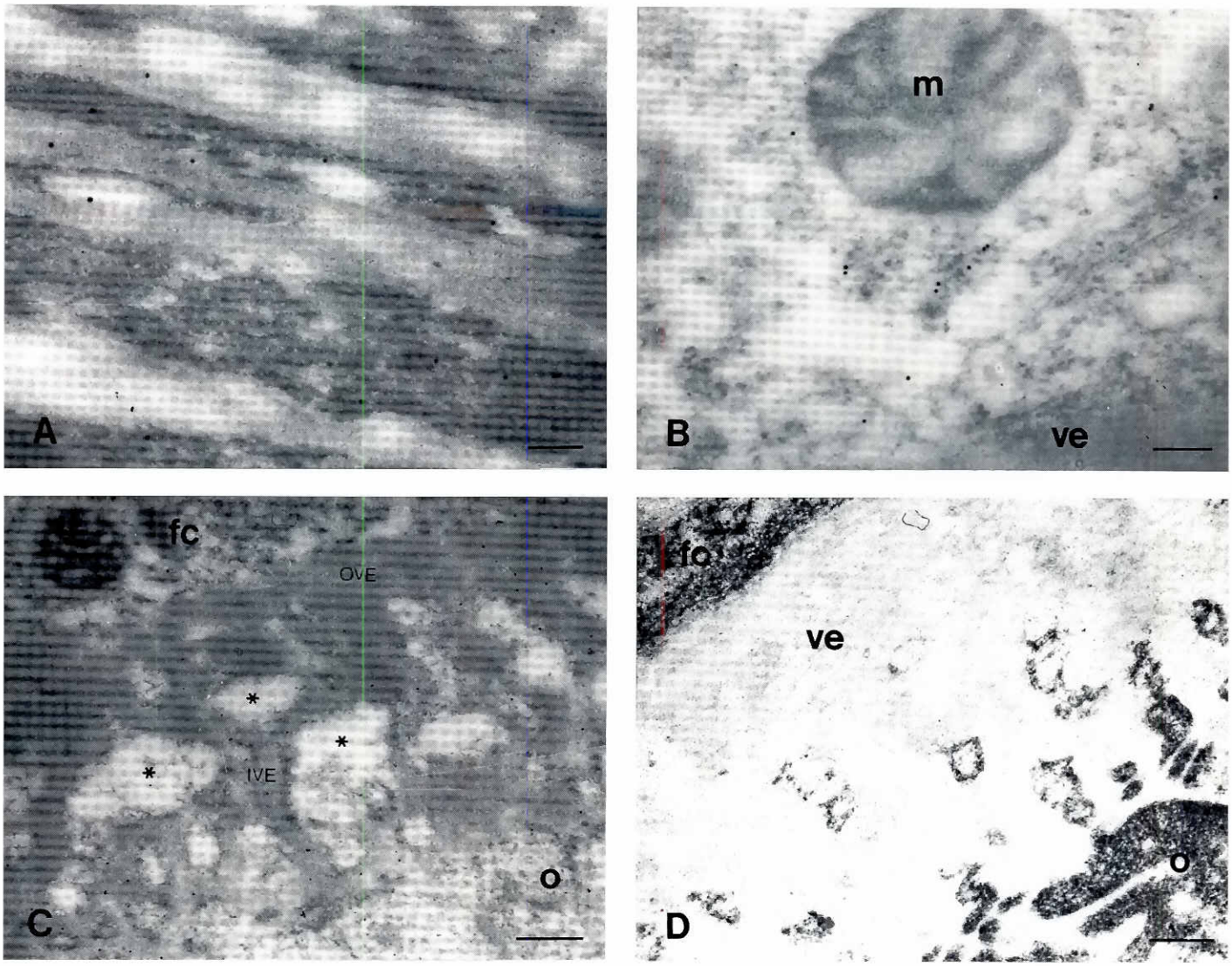

Fig. 6. Immunoelectron microscopy of oocyte investing envelopes. (A) *Theca layer: few gold particles can be seen on this envelope.* **(B)** *Follicle cell layer: gold particles can be seen on follicle cell cytoplasm. Note that mitochondria (m) is not gold-labeled; ve: vitelline envelope.* **(C)** *Vitelline envelope: vitronectin-like protein is immunolocalized both in the inner and outer vitelline envelope, while tunnels (asterisks) are poorly labeled; o: oocyte, fc: follicle cell, IVE: inner vitelline envelope, OVE: outer vitelline envelope.* **(D)** *Control ultrathin section treated with anti-human vitronectin antibody preadsorbed with an excess amount of human vitronectin; o: oocyte, fc: follicle cell, ve: vitelline envelope. Bars: A,B, 0.1 μm; C,D, 0.4 μm.*

mass, number of bands in SDS-PAGE and/or carbohydrate composition (Kitagaki-Ogawa *et al.*, 1990; Nakashima *et al.*, 1992).

Most vitronectins described cross-reacted with antibodies raised against human vitronectin (Kitagaki-Ogawa *et al.*, 1990); thus, these antibodies proved to serve as a useful tool to detect vitronectins from other sources.

Human VN and *Bufo* vitronectin-like protein were purified from serum by the same method. They have two biological properties in common: heparin-binding activity and an RGD-dependent cell-spreading activity. SDS-PAGE of human VN and *Bufo* vitronectin-like protein revealed that each protein consists of two polypeptides of 65-75 kDa and 64-72 kDa, respectively. Moreover, Nakashima *et al.* (1992) reported that VN from *Xenopus* appeared as a broad band with Mr of 58-63 kDa. The immunoblotting analyses for *Bufo* vitronectin-like protein demonstrated that this protein reacts with two monoclonal antibodies raised against human VN, but it was not detected by the antibodies preadsorbed with an excess amount of human VN. These results indicate that *Bufo* vitronectin-like protein has immunological cross-reactivity with human VN. They are in agreement with most studies for animal vitronectins (Kitagaki-Ogawa *et al.*, 1990; Miyazaki *et al.*, 1992; Nakashima *et al.*, 1992).

There have been several reports describing the presence of VN in different tissues and body fluids (Seiffert *et al.*, 1993). Interestingly, yolk vitronectin was well isolated and characterized from chicken eggs (Nagano *et al.*, 1992), but to our knowledge, this is the first report that analyzes the presence of VN during oogenesis, a relevant process that progressively leads from oogonia to a mature oocyte. On the basis of cytological analysis, we have shown that vitronectin-like protein was preferentially located in growing oocyte cytoplasm throughout oogenesis.

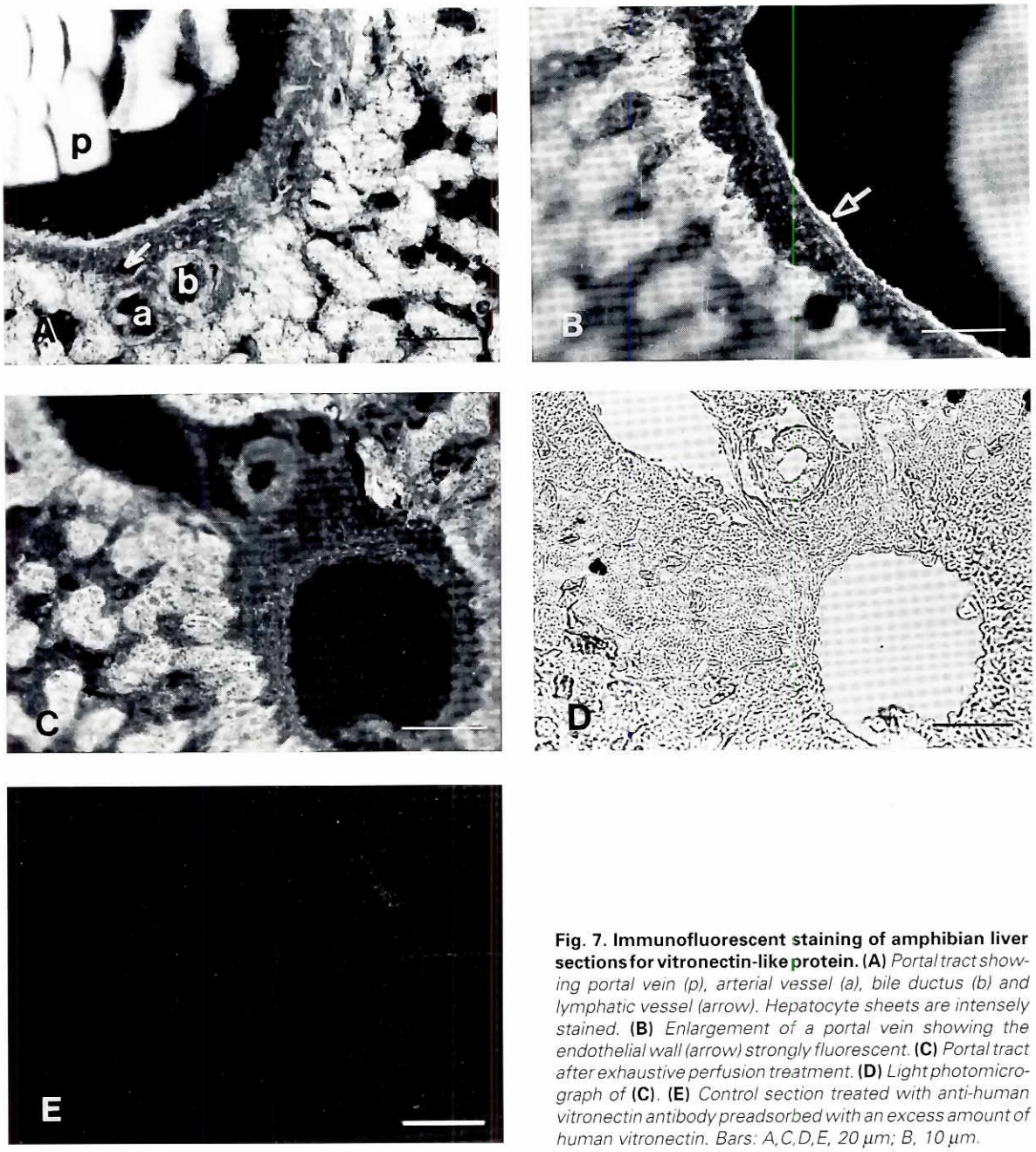

Fig. 7. Immunofluorescent staining of amphibian liver sections for vitronectin-like protein. (A) *Portal tract showing portal vein (p), arterial vessel (a), bile ductus (b) and lymphatic vessel (arrow). Hepatocyte sheets are intensely stained.* **(B)** *Enlargement of a portal vein showing the endothelial wall (arrow) strongly fluorescent.* **(C)** *Portal tract after exhaustive perfusion treatment.* **(D)** *Light photomicrograph of* **(C)**. **(E)** *Control section treated with anti-human vitronectin antibody preadsorbed with an excess amount of human vitronectin. Bars: A,C,D,E, 20 μm; B, 10 μm.*

We observed a different labeling pattern according to the stages under consideration. Thus, this study provides the evidence that cortical oocyte cytoplasm increased its immunoreactivity to anti-human VN antibody beginning at stage III parallel to the vitellogenic process. The process of yolk synthesis is perhaps the most intensively studied aspect of oogenesis. The internalization of substances into yolk organelles of telolecithal oocytes is an important feature of egg-laying animals. Thus, the incorporation, transport and deposition of vitellogenin have been well established (Richter and Bauer, 1990).

The ultrastructure of the vitellogenic process in anuran amphibia has been previously reported. Wallace (Wallace and Dumont, 1968; Wallace, 1985) first reported the transport pathway of vitellogenin in vitellogenic oocytes. Incorporation and transport pathway of yolk precursor vitellogenin was shown in *Xenopus laevis* (Bauer and Richter, 1990; Richter and Bauer, 1990), in *Bufo marinus* (Richter, 1987) and in *Bufo arenarum* and *Ceratophrys cranwelli* (E. Villecco, personal communication). The vitellogenin pathway involves several cortical structures: coated pits, coated vesicles, uncoated vesicles, tubular endosomes, multivesicular bodies and primordial yolk platelets. Multivesicular bodies (MVB) in particular are differentiated during the initial phase of oocyte growth. It has been suggested that they might be involved in processing and carrying the endocytosed yolk constituents. Fur-

thermore, the passage via MVB is required for the formation of the mature yolk platelet (Wall and Patel, 1987a,b). The presence of vitronectin-like protein in MVB and in primordial yolk platelets strongly suggests that this protein is closely related to yolk platelets formation. However, further studies are necessary to establish the mechanism by which vitronectin-like protein is incorporated by the surface of oocytes from circulation.

On the basis of immunofluorescent or immunoelectron microscopy analyses we observed the labeling of the theca layer, follicle cells and vitelline envelope suggesting an unidirectional transport pathway. Vitronectin-like protein could be transported via the blood stream to the ovary, where it would successively pass through the endothelium, the basal lamina, the follicular epithelium and the vitelline envelope to reach its final destination: the yolk platelets in the oocyte.

Although the role of VN during oogenesis is not known, it is possible that VN could act as an adhesive material during morphogenesis. With regard to this point, previous studies have shown that a yolk-granule component of full-grown ovarian oocytes of the newt acts as an adhesive material for dissociated gastrula cells (Komazaki, 1987). In addition, the presence of VN during amphibian oogenesis may probably be related to the fact that other adhesion-mediating proteins such as fibronectin (Darribere *et al.*, 1984), laminin (Riou *et al.*, 1987) and cadherin (Ginsberg *et al.*, 1991) have been detected during amphibian oogenesis and play a role during morphogenesis. In this context, the presence of vitronectin-like protein in yolk platelet probably plays a role with other cell adhesion proteins as was suggested for avian yolk VN by Nagano *et al.* (1992). However, further specific biochemical studies are required to determine whether the vitronectin-like protein found in yolk platelets is similar to that of serum.

TABLE 2

EFFECT OF ESTROGEN ON SERUM VITRONECTIN-LIKE PROTEIN LEVELS IN ADULT *BUFO ARENARUM*

Animals	Treatment	Mean concentration (μg/ml)
Female	—	181.5±16.6
Male	—	127.8±11.6
Female	Estrogen	381.1±18.2
Male	Estrogen	320.7±11.0

Dose: estrogen (17-ß-estradiol), 100 μg/100 g body weight. Serum was collected from 10 animals in each case and assayed for VN as indicated in Materials and Methods. The values represent the mean ±S.D.

Limited information is available on the tissues that produce VN *in vivo*. *In situ* hybridization assays revealed that hepatocytes are the main source of VN biosynthesis *in vivo* (Seiffert *et al.*, 1991) and several hepatocarcinoma cell lines have been shown to synthesize VN *in vitro* (Barnes and Reing, 1985; Yasumitsu *et al.*, 1993). In this study we have shown positive immunostaining in liver sections. Probably VN could be synthesized and secreted to circulation and selectively taken up by vitellogenic oocytes in a way similar to the vitellogenin in *Xenopus* (Wallace, 1978; Yoshizaki, 1992), fish (Hamazaki *et al.*, 1987), chicken (Stifani *et al.*, 1990).

The inflammatory stimuli produced by endotoxin, Freund complete adjuvant and turpentine, which increase the serum levels of interleukin-6 and corticosterone, enhance the expression of hepatic VN mRNA. These results indicate that VN in the rat liver is regulated as an acute-phase protein (Seiffert *et al.*, 1993).

In amphibians, it has been well established that the amphibian reproductive cycle is under hormonal control (Redshaw, 1972). The yolk proteins synthesis (e.g. vitellogenins) in liver is under the control of estrogen (Clemens, 1974; Wallace and Bergink, 1974). In order to explore the possibility that the synthesis of VN is hormonally regulated, we measured serum vitronectin-like protein concentration in control adult and in estrogen-treated adult animals. There was a significant increase in the mean serum vitronectin-like protein concentration in the adult animals (males and females) treated with estradiol 17-ß. Interestingly enough, no significant sex difference was observed. In males, although serum vitronectin-like protein concentration responded to estrogen injection, the physiological mechanism of regulation is unknown and this point may be investigated. Our results have confirmed that in non-mammalian vertebrates, the liver-related events are involved in oogenesis (Wallace, 1985).

Yolk platelets are the most abundant organelles in the oocyte. It is believed that the yolk platelets provide the organic components and/or precursors that are utilized during events of morphogenesis and differentiation. Amphibian eggs contain variable amounts of yolk. Although yolk serves as an energy source during embryogenesis, it is also apparent that it plays a role in developmental events by supplying nutritive and informational essential substances, precursors, templates, structural molecules or tissue inducing substances (Carson and Lennarz, 1979; Eckelbarger, 1986) that are utilized after oocyte activation for the support of whole embryogenesis. The specific biological function of the vitronectin-like protein stored in yolk platelets is unknown and requires further studies.

Materials and Methods

Animals

Adult specimens of *Bufo arenarum* Hensel, 100-150 g body weight, were collected in Famaillá, Tucumán, Argentina, in winter (July, August, September) and kept in a moist chamber at 17°C for a week before each experiment.

Serum

Blood drawing from adult *Bufo arenarum* was performed by cardiac puncture. After collection, blood was allowed to clot at room temperature. Serum was taken in a small amount of ice-cold preparation buffer (PBS containing 0.5 mg/ml phenylmethylsulfonyl fluoride, pH 7.4). This solution was centrifuged at 24,000g for 20 min and stored at -20°C in small aliquots until use. The supernatant, subjected to analysis, is termed blood serum sample hereafter.

Isolation of Bufo arenarum vitronectin-like protein

Both amphibian and human vitronectin were isolated by heparin affinity chromatography using the buffer conditions according to the method of Yatohgo *et al.* (1988). Serum sample was applied to precolumns of Sepharose 4B and heparin-Sepharose equilibrated with 10 mM Na-phosphate buffer, pH 7.7, containing 0.13 M NaCl and 5 mM EDTA. The flow-through fractions were pooled and treated with urea to a final concentration of 8 M for 2 h and then applied to a heparin-Sepharose column equilibrated with 10 mM Na-phosphate buffer, pH 7.7, containing 0.13 M NaCl, 5 mM EDTA and 8 M urea. After the column had been successively washed both with the same buffer and with the same buffer plus 10 mM 2-mercaptoethanol, vitronectin was eluted with 10 mM Na-phosphate buffer, pH 7.7, containing 0.5 M NaCl, 5 mM EDTA and 8 M urea. The eluate was pooled, dyalized, lyophilized and stored until use at -20°C.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting procedure

Denaturing polyacrylamide slab gel electrophoresis was carried out following Laemmli's procedure (Laemmli, 1970). Sodium dodecylsulfate denatured proteins were electrophoresed in 7.5% polyacrilamide gels. Samples were boiled for 3 min in 2% SDS, 2% 2-mercaptoethanol, 20 mM Tris-HCl, pH 7.0. The molecular weights of the denatured samples were estimated from a calibration curve obtained with the standard proteins in an Prestained SDS-PAGE Standard solution (Sigma Chemical Co.). The gels were stained with Coomasie Blue R-250. Protein determination was performed by the method of Lowry *et al.* (1951).

After SDS-PAGE, the immunoblotting analyses were performed according to the method of Towbin (1979). Electrophoresed proteins were transferred from the gel to a nitrocellulose membrane that was rinsed with distilled water and blocked with 3% BSA-PBS for 1 h at room temperature. A 1:300 dilution of the mouse monoclonal anti-human vitronectin IgM (Clone VIT-2, Sigma Chemical Co.) or mouse monoclonal anti-human vitronectin IgG (Clone VN5-3, Panvera Corp., WI, USA) was added and incubated overnight at 4°C. Then the membrane was rinsed three times for 10 min with PBS with gentle shaking. The antigen-antibody reaction was detected using biotin-conjugated anti-mouse polyvalent immunoglobulins and ExtrAvidin-Peroxidase conjugate (Sigma Chemical Co.), each diluted 1:1000 and incubated for 2 h at room temperature. Between these incubations and after them, the membrane was rinsed with PBS three times for 10 min. Peroxidase activity was detected by incubating blots in 3,3'-diaminobenzidine-H_2O_2.

Cell-spreading assay

Cell-spreading activity was determined using BHK cells in Grinnell's medium (150 mM NaCl, 1 mM $CaCl_2$, 3 mM KCl, 0.5 mM $MgCl_2$, 6 mM Na_2HPO_4, and 1 mM KH_2PO_4, pH 7.3). Polystyrene 96-well tissue culture plates (Nunc) were coated with *Bufo* vitronectin-like protein, human vitronectin, or bovine serum albumin at different concentrations in the adhesion medium for 1 h at 37°C. Unbound protein was removed. BHK cells suspended in the adhesion medium were added to the wells ($1x10^4$ cells in 0.10 ml/well). After 90 min at 37°C, cells were fixed with phosphate-buffered saline containing 2% glutaraldehyde, 4% formaldehyde, and 5% sucrose and the percentage of spread cells was counted under a microscope. Inhibition assays by different antibodies against vitronectin or RGD-containing synthetic peptides were carried out on the wells coated with 1 µg/ml *Bufo* vitronectin-like protein, human vitronectin or fibronectin. Antibodies, GRGDSP or GRGESP peptides were added to the cell suspension on the well at different concentrations.

Immunofluorescence microscopy

The localization of immunoreactive substances in sections of the ovary and the liver of males, females and estrogenized males (50-100 µg/100 g body weight) of adult *Bufo arenarum* specimens was determined by an indirect immunofluorescence method. Specimens were fixed in Bouin's solution, dehydrated through a graduated alcohol series and embedded in paraplast. The sections were cut at 5 µm thickness, deparaffinized and rinsed with PBS (pH 7.4). Then they were blocked with 3% bovine serum albumin-PBS for 3 h at room temperature. After blocking they were treated with a 1:120 dilution of anti-human VN antibodies (mouse monoclonal anti-human vitronectin IgM, Clone VIT-2, Sigma Chemical Co., or mouse monoclonal anti-human vitronectin IgG, Clone VN5-3, Panvera Corp., USA) or with anti-human VN antibodies previously preadsorbed with human VN and incubated at 4°C in a moist chamber overnight. After rinsing with PBS the sections were treated with 1:100 dilution of FITC-conjugated rabbit anti-mouse IgG (Sigma Chemical Co.) and incubated at 4°C in a moist chamber for 2 h. Slides were mounted in Mowiol (Hoechst), observed with an Nikon Fluophot epifluorescence microscope, and photographed on Ilford HP5 plus (ASA 400). All negatives were exposed and printed identically, so that relative differences reflected variations in the staining intensities of the specimens.

Immunoelectron microscopy

The ovary of the *Bufo arenarum* female was fixed with 4.0% formaldehyde and 0.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) at 0°C for 3 h. Then it was rinsed with several changes of PBS (pH 7.2), dehydrated by an alcohol series and embedded in LR white. The sections were cut on a Sorvall Porter Blum MT1 ultramicrotome and mounted on uncoated nickel grids. For immunogold cytochemistry, grids were floated on a drop of filtered 2% BSA in PBS for 10 min and transferred without washing to a drop of primary antibodies (Clone VIT-2, Sigma Chemical Co., and Clone VN5-3, Panvera Corp., USA) diluted 1:120 with PBS for 2 h at room temperature. Control grids were floated on human VN preadsorbed anti human VN antibody, and the effect of the second antibody alone was also tested. In order to indicate the intensity and location of an established marker, we used an anti-histone (Boehringer, Germany) monoclonal mouse antibody (clone 1B3), which is presumed to be reactive only with the nucleus. Gold particles were observed on the nucleus, while no immunoreactivity was detected in the cytoplasm or in any other organelle (Fig. 5D, inset). After washing with filtered PBS, each grid was floated on successive drops of biotin-conjugated anti-mouse polyvalent immunoglobulins (affinity isolated antibodies developed in goats, Sigma Chemical Co., St. Louis, MO) and ExtrAvidin-10 nm Colloidal gold labeled (Sigma Chemical Co.), each diluted 1:1000 and incubated 2 h at room temperature. Final washing was carried out with filtered PBS and blotted with filter paper. Grids were stained with uranyl acetate and lead citrate and examined with a Zeiss EM 109 transmission electron microscope. Figures 5 and 6 show the immunolocalization of vitronectin-like protein using anti-human vitronectin antibody (Clone VIT-2, Sigma Chemical Company).

Estimation of vitronectin concentration

In order to quantitate serum vitronectin, we coated polystyrene 96-well plates for ELISA (product M4034, Sigma Chemical Co., St. Louis, MO, USA) as described by Miyamoto *et al.* (1989). Amphibian serum was diluted to 5% in phosphate-buffered saline containing 0.1% SDS, and boiled for 5 min. Polystyrene plates were incubated each with 50 µl of SDS-treated serum, untreated serum, and vitronectin standard (VN purified from human plasma using a heparin affinity column according to Yatohgo *et al.* (1988) at room temperature for 1 h). After washing with PBS three times, plates were incubated with 3% (w/v) bovine serum albumin in PBS (BSA-PBS) at room temperature for 1 h. After rinsing with PBS 5 times, plates were incubated with mouse monoclonal anti-human vitronectin IgM diluted at 1:350 in PBS containing 1% BSA at room temperature for 1 h or with pre-immune mouse serum under the same conditions. After rinsing 5 times, plates were incubated with a biotin-conjugated anti-mouse polyvalent immunoglobulin diluted 1:1000 at room temperature for 1 h, and then incubated with ExtrAvidin-Peroxidase conjugate (Sigma Chemical Co.) diluted 1:1000 at room temperature for 1 h. Finally, plates were rinsed 5 times with PBS and incubated at room temperature with 100 µl of 0.1 M citric acid, 0.4 mg/ml o-phenylendiamine, 2.5 mM H_2O_2 and 0.2 M Na_2HPO_4. After 15 min reaction was stopped with 50 µl of 2N H_2SO_4. The adsorbed VN on the plates was quantitated by the Absorbance at 492 nm.

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