Characterization of a Maternal Type VI Collagen in *Xenopus* Embryos Suggests a Role for Collagen in Gastrulation

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Abstract. We characterized a novel extracellular matrix element that is present in the earliest developmental stages of *Xenopus laevis*, and is recognized by an mAb 3D7. Based on amino acid composition, breakdown patterns by bacterial collagenases, and the molecular weights of the components of the antigen (240, 200, and 140 kD), we found it very similar to mammalian collagen type VI. The antigen is evenly distributed in unfertilized eggs. Shortly after fertilization, it becomes localized intracellularly in the periphery of the cleaving embryo as well as in the extracellular spaces. During gastrulation, the antigen was localized in the cells lining the blastopore and in the extracellular space between the two cell layers, in the presumptive archenteron. When Fab elements of the 3D7 antibody were added to the culture medium, gastrulation was blocked, suggesting a role for the antigen in gastrulation movements.

THE extracellular matrix plays an important role in cell interactions. It has become clear that the extracellular matrix is not only involved in the structural organization of cells into tissues. Important components such as fibronectin, laminin and collagens influence the adhesiveness of cells and their capacity to migrate and differentiate. As these events occur extensively in early embryos, it is not surprising that the extracellular matrix has an important role in early embryogenesis (for a review see Thiery, 1989).

In amphibian embryos, where morphogenetic movements have been well characterized (Gerhart and Keller, 1986; Keller, 1986), the role of fibronectin has been studied extensively. The amount of fibronectin increases during gastrulation and this matrix element is found coating the ectoderm cells over which the involuting mesoderm will migrate (Lee et al., 1984; Nakatsuji et al., 1985). Antibodies against fibronectin prevent gastrulation (Boucaut et al., 1984*a*). Fibronectin is also responsible for directing the migration of neural crest cells (Boucaut et al., 1984*b*).

The role of fibronectin in gastrulation involves the fibronectin receptor (Duband et al., 1986; Darribère et al., 1988). Antibodies against the fibronectin receptor complex arrest gastrulation when injected into the blastocoel of early gastrulae (Darribère et al., 1988). The localization and timing of synthesis of laminin, another major extracellular matrix element, is similar to that of fibronectin (Darribère et al., 1987). The only thing known about a third class of extracellular matrix elements, collagens, is that synthesis starts after neurulation (Green et al., 1968). Whether maternal collagens have an earlier role is unknown.

It must be realized, however, that gastrulation, where both fibronectin and laminin are involved, is a relatively late event. Important morphogenetic events such as blastocoel formation, epiboly, changes in cell adhesiveness and rapid cell divisions precede gastrulation. So far, however, no extracellular matrix elements that are involved in these early events have been characterized. A galactoside-binding lectin was found in the *Xenopus* cleavage stages (Roberson and Barondes, 1982; 1983) and was found to be secreted into the extracellular matrix (Outenreath et al., 1988) but the function of this lectin is unknown.

In this paper, we report a novel extracellular matrix element, detected by an mAb, that is found in the earliest stages of *Xenopus laevis* development. After fertilization, the antigen becomes localized in extracellular spaces and also intracellularly, in the periphery of the cleaving embryo. The antigen shows many of the characteristics of collagen type VI. Functional studies suggest that it may be involved in regulating cell movements during gastrulation.

Materials and Methods

Culture Conditions

Embryos were obtained via natural or artificial fertilization by standard procedures. Embryonic stages were determined according to Nieuwkoop and Faber (1967). Embryos were dejellied in 2% cysteine hydrochloride (Sigma Chemical Co., St. Louis, MO) (pH 8.0) and then cultured in MMR[1] (0.1 M NaCl, 2 mM KCl, 1.0 mM $MgSO_4$, 2.0 mM $CaCl_2$, 5.0 mM Hepes, and 0.1 mM EDTA [pH 7.8]).

1. *Abbreviations used in this paper*: MMR, 0.1 M NaCl, 2 mM KCl, 1.0 mM $MgSO_4$, 2.0 mM $CaCl_2$, 5.0 Hepes, and 0.1 mM EDTA (pH 7.8); NGS, normal goat serum.

Production of mAbs

The hybridoma-secreting mAb 3D7 was produced by fusion of splenocytes with the myeloma cell line Sp2/0-Ag14 (Schulman et al., 1987) using published procedures (Fazekas de St. Groth and Scheidegger, 1980). BALB/c mice were immunized with 3% paraformaldehyde-fixed late-blastula stage embryos. The fixed embryos were homogenized, centrifuged for 30 min at 10,000 *g* and ~5 mg of the supernatant was used in a 1:1 dilution with complete Freund's adjuvant for immunization. At 2 and 4 wk after the first injection, the mice were boosted with the same material, in incomplete Freund's adjuvant. Animals with the highest antiserum titers received a final intraperitoneal booster. 4 d later, splenocytes were harvested from these animals and fused with the myeloma cell line. Hybridomas that gave a positive reaction were subcloned at least twice by limiting dilution. Screening for a positive reaction was done using an ELISA (see below) on a cell homogenate from blastula embryos. Ascites fluid was produced by injecting 1-2.5 × 10^7 cells into pristane-primed BALB/c mice and collecting fluid 15-30 d later. The immunoglobulin containing fraction of the ascites fluid was prepared by precipitation with 50% ammonium sulfate, followed by dialysis overnight against PBS, and purified by protein A-Sepharose chromatography (Pharmacia Fine Chemicals, Uppsala, Sweden). The antibody was eluted using 0.1 M citric acid (pH 4.0) (see product information on protein A-Sepharose, Pharmacia Fine Chemicals). Monovalent antibodies were obtained using a procedure described by Matthew and Reichardt (1982). The resulting monovalent antibodies were purified using a protein A-Sepharose column that retained Fc fragments as well as intact antibodies.

ELISA, Western Blotting, and Affinity Chromatography

Cell homogenates of embryos were centrifuged for 10 min at 1,000 *g*. 10-μg aliquots of the supernatant were coated in 96-well plates (Greiner Alphen a/d Rijn, The Netherlands) overnight at 37°C. Aspecific binding was blocked using 10% normal goat serum (NGS) in PBS with 0.05% Tween-20 (Sigma Chemical Co., St. Louis, MO) (buffer A). Dilution of the 3D7 antibody was in buffer A (0.1 mg/ml) and of the second antibody goat anti-mouse (IgM-Fc)/PO (Nordic Immunology, Tilburg, The Netherlands) (1:1,000 in buffer A). Orthophenyl diamine was used as substrate for the peroxidase reaction. The amount of colour development was measured at 492 nm in a Multiscan (Titertek, Elfab Oys Finland).

SDS-PAGE was carried out on 5-15% polyacrylamide gels according to the procedure of Laemmli (1970) using myosin (200 kD), β-galactosidase (166 kD), phosphorylase B (92 kD), BSA (66 kD), and ovalbumin (45 kD) as molecular mass markers. Transfer of proteins to nitrocellulose (BA 85, 0.45 μm; Schleicher & Schuell, Dassel, FRG) was performed by electrophoresis at 30 V for 16 h at 4°C in a buffer containing 192 mM glycine, 25 mM Tris, and 20% methanol as described by Towbin et al. (1979). Blots were incubated in PBS/0.05% Tween-20 and 10% NGS(A) for 1 h at room temperature to block a specific binding. 3D7 antibody was used in buffer A (0.5 mg/ml). Washes were done with PBS/0.05% Tween-20. GAM (IgM-Fc)/PO (Nordic Immunology) was diluted in a 1:1,000 dilution as the second antibody. The peroxidase substrate was tetra-methyl benzidine. Silver staining of the gels to detect the purified antigen was performed according to the procedure of Merrill et al. (1981).

Purified 3D7 antibody was coupled to cyanogen bromide-activated Sepharose (Pharmacia Fine Chemicals, Uppsala) following procedures given as product information by Pharmacia. The coupled antigen was eluted using 1 M acetic acid. After elution, the fractions were dialyzed overnight against H_2O at 4°C.

Secreted antigen was obtained by placing 30 stage 2 embryos for 4 h in 1 ml MMR containing protease inhibitors (5 μg/ml aprotinin, 10 mM benzamidine, 2 mM PMSF, 5 mg/ml leupeptin, and 5 mg/ml pepstatin). Great care was taken that embryos were not damaged. The medium was collected, dialyzed overnight against H_2O at 4°C, and freeze-dried. The concentrated product was analyzed by SDS-PAGE and Western blotting.

Collagenase and Pepsin Digestion

Collagenase I and VII (Sigma Chemical Co.) at concentrations of 75 and 125 U/ml, respectively, were incubated with cell homogenate, secreted product or purified antigen in 50 mM $CaCl_2$, 400 mM NaCl, 50 mM Tris-HCl, (pH 7.4), 5 mM PMSF and the other protease inhibitors as indicated before for 4 or 12 h at room temperature. Controls were incubated under the same conditions and period in the absence of collagenases. In parallel incubations, the same amount of cell homogenate or the molecular weight markers were incubated with the collagenases. The blots of these preparations were stained with Coommassie brilliant blue to check whether aspecific proteolysis had occurred. This was not the case.

Pepsin digestion (1 mg/ml; Sigma Chemical Co.) was performed in 0.5 M acetic acid for 12 h at 4°C.

Amino Acid Analysis

Purified antigen was hydrolysed under vacuum in 6 N HCl (16 h, 110°C) and amino acid analysis was carried out by HPLC using the Waters Pico-Tag system (Waters Instruments, Millipore Corp., Bedford, MA).

Immunocytochemistry

Embryos were fixed in 0.1% glutaraldehyde/2% paraformaldehyde for 6 h at room temperature. After fixation, embryos were washed with PBS, dehydrated with ethanol and paraffin embedded. Paraffin blocks were stored at 4°C. Sections were cut on a Spencer 820 microtome (Lameris, Utrecht, The Netherlands), collected on protein-glycerin-coated glass slides and dried at 37°C for 24 h. Sections were deparaffined with xylene and ethanol. Incubation with 3D7 antibody (0.1 mg/ml) for 60 min at 37°C was followed by three washes in PBS before incubation with a 1:50 dilution of GAM/IgM(Fc)/FITC (Nordic Immunology) for 60 min at 37°C. Slides were washed with PBS, mounted in PBS glycerol 1:1 and examined using a fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY).

In control experiments, cryostat sections of unfixed or methanol fixed embryos were used. The localizations found were the same, but the quality of the sections was poor and the background immunofluorescence was high. Of all fixation procedures we tried, the glutaraldehyde/paraformaldehyde fixation combined with paraffin embedding gave the best signal to background ratio.

Results

mAbs were raised against a crude cell homogenate from paraformaldehyde fixed late-blastula stage *Xenopus laevis* embryos. Hybridoma supernatants were screened on a homogenate of *Xenopus* pregastrula stage embryos, using an ELISA. Supernatants that reacted positively were tested further by immunocytochemistry, using paraffin-embedded sections of 0.1% glutaraldehyde and 2% paraformaldehyde-fixed embryos. We selected antibodies that gave staining of (*a*) pregastrula embryos and (*b*) extracellular material. One of the antibodies (3D7), that was characterized as an IgM, met both criteria, and was used for further studies.

Biochemical Characterization of the 3D7 Antigen

The antigen was characterized using an ELISA and Western blotting. Embryos were homogenized and the homogenate was spun at 10,000 *g* for 10 min. The supernatant was used either used to coat wells for an ELISA or to run on a 5-15% SDS-PAGE gel, followed by blotting on a nitrocellulose filter. Both techniques showed that the antigen recognized by 3D7 is present in the unfertilized egg and in early embryonic stages. No dramatic quantitative changes nor changes in the molecular weights of the components of the antigen were observed between unfertilized eggs and different early embryonic stages (results not shown). The following results were obtained using homogenates obtained from a mixture of gastrula and cleavage stages.

The antibody recognized three components, with molecular masses of 240, 200, and 140 kD (Fig. 1 *A*, lane *1*). There was no detectable nonspecific background staining as checked using the second peroxidase-labeled antibody only (Fig. 1 *A*, lane *4*) or a control IgM mAb that was made against the mouse EGF receptor. This mouse monoclonal antibody did not cross-react with *Xenopus* material. The pe-

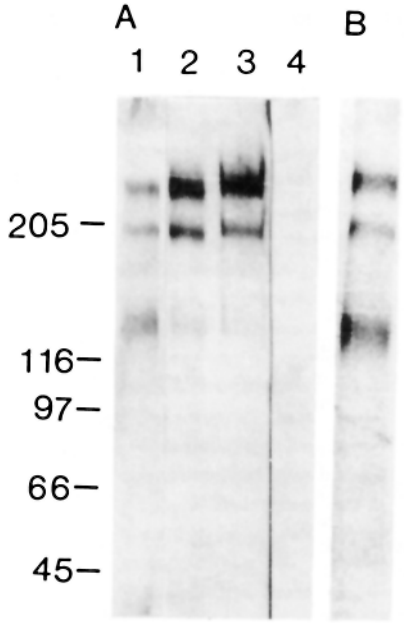

Figure 1. Analysis of 3D7 antigen using SDS-PAGE and Western blotting or silver staining. (*A*) A blastula cell homogenate (10 μg) (lane *1*) was subjected to 10 min at 10,000-*g* centrifugation to remove yolk platelets, and the supernatant was then resolved by SDS-PAGE (5–15% polyacrylamide gradient gel), transferred to nitrocellulose, and immunostained using the 3D7 mAb (lanes *1–3*). As negative control the second peroxidase-labeled antibody only was used (lane *4*). Lane *2* shows immunoaffinity-purified antigen. Lane *3* shows concentrated culture medium containing secreted antigen. Binding of the antibodies was visualized using a peroxidase-conjugated second antibody. (*B*) SDS-PAGE of immunoaffinity-purified 3D7 antigen. Antigen was obtained from blastula cell homogenate as in *A*. Proteins were visualized by silver staining of the gel. The positions of the molecular weight markers are indicated to the left of the panels.

ripheral localization of the antigen in the cleavage stages (see below) suggested to us that it might be secreted. To test this, we cultured eggs from which the jelly coat had been removed, for 6 h in a medium containing protease inhibitors. The dialyzed and freeze-dried medium was subsequently tested for the presence of antigen. We indeed found that the antigen was present in the external medium, but, surprisingly, that the secreted antigen consisted almost exclusively of the 240- and 200-kD components (Fig. 1 *A*, lane *3*). When the embryos were not dejellied, no antigen was found in the medium.

To characterize the antigen further and to be able to determine its amino acid composition, we purified it. We immunopurified the antigen using CNBr-Sepharose-coupled 3D7 antibody. The coupled antibody was incubated for 16 h at 4°C with a cell homogenate. After washing extensively, the antigen was eluted with 1 M acetic acid. The purity of the antigen was checked by silver staining after SDS-PAGE electrophoresis (Fig. 1 *B*). The 240-, 200-, and 140-kD components were detected both by silver staining (Fig. 1 *B*) and Western blotting (Fig. 1 *A*, lane *2*).

The 3D7 Antigen Might Be Collagen VI

The molecular weights, time of appearance, and localization of the antigen recognized by 3D7 are unlike those of known extracellular matrix components that have been characterized in early *Xenopus* development; i.e., fibronectin (Lee et al., 1984) and laminin (Darribère et al., 1987). There is, however, a resemblance in molecular weights between our antigen and the type VI collagen (Heller-Harrison and Carter, 1984; Hessle and Engvall, 1984; Trüeb and Bornstein, 1984; Engel et al., 1985; Trüeb and Winterhalter, 1986). The last three groups also found secreted components of 200 and 240 kD in the culture media. We therefore tested the effects of bacterial collagenases on the 3D7 antigen. Under the conditions used (4 h at room temperature), collagen I was completely degraded, whereas neither a ladder of marker proteins nor a ladder of *Xenopus* proteins were affected (results not shown). The results (Fig. 2) show that collagenase I treatment caused disappearance of the 200-, 240-, and 140-kD components (lanes *2* and *4*). This degradation occurred both in cell homogenate (lanes *1* and *2*), and in CNBr-Sepharose, purified antigen (lanes *3* and *4*). New bands of ~75 and 100 kD and a cluster of bands at 40–50 kD also appeared. Breakdown products of the same size have also been found by others (Trüeb and Bornstein, 1984). The 40–50-kD cluster is especially interesting, since these bands could be so-called short-chain collagen, which is considered to be a breakdown product of the native higher molecular weight components (Heller-Harrison and Carter, 1984; Timpl

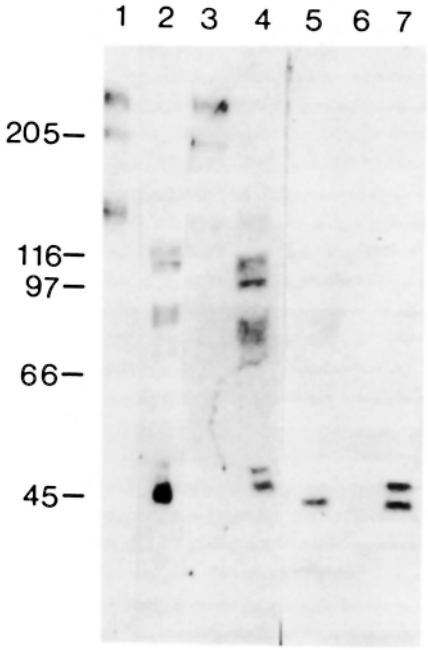

Figure 2. Digestion of 3D7 antigen using collagenases. Blastula supernatant (lane *1*) or purified antigen (lane *3*) were incubated with 75 U/ml bacterial collagenase type I (Sigma Chemical Co.) (lanes *2* and *4*) for 4 or 12 h (lane *5*) at RT before SDS-PAGE and Western blotting. Blastula supernatant was also incubated with bacterial collagenase type I for 12 h at room temperature in the presence of 1 M DTT (lane *6*). Further blastula supernatant was incubated with 0.5 mg/ml pepsin (Sigma Chemical Co.) at 4°C 12 h (lane *7*). Binding of the antibodies was visualized using a peroxidase-conjugated second antibody. The results were obtained using different gels. The positions of molecular weight markers are indicated to the left of the panels.

Table I. Amino Acid Composition of the Purified Antigen Recognized by mAb 3D7

Amino acid	Residues/1,000 residues
Aspartic acid	81
Glutamic acid	120
Hydroxyproline	36
Serine	46
Glycine	170
Histidine	22
Arginine	29
Threonine	32
Alanine	36
Proline	88
Tyrosine	38
Valine	61
Methionine	28
Cysteine	28
Isoleucine	22
Leucine	57
Hydroxylysine	34
Phenylalanine	32
Lysine	40

and Engel, 1987). When the purified antigen was incubated with collagenase I for 12 h at room temperature, only the 45-kD band remained (lane *5*).

Partial resistance to bacterial collagenases and the presence of a cluster of lower molecular weight components have been reported by others as a specific property of collagen VI (Heller-Harrison and Carter, 1984; Trüeb and Bornstein, 1984; Trüeb and Winterhalter, 1986). These components were, however, sensitive to bacterial collagenases under reducing conditions (with 1 M DTT) (Heller-Harrison and Carter, 1984). We found the same to be true of the 3D7 antigen. The antigens were digested completely by collagenase I (Fig. 2, lane *6*), in the presence of 1 M DTT. Addition of 1 M DTT alone failed to affect the antigen (results not shown). Another resemblance to collagen VI was found after pepsin digestion. The higher molecular weight components were degraded completely and the 40–47-kD components were found to be enriched (lane *7*), a characteristic of collagen VI (Heller-Harrison and Carter, 1984; Timpl and Engel, 1987).

We established the amino acid composition of the purified antigen by using immuno-affinity chromatography via the 3D7 antibody (see above). The purity of the antigen was checked by silver staining after PAGE (Fig. 1 *B*). As shown in Table I, we detected both hydroxyproline and hydroxylysine, two amino acids that are characteristic for collagens. The high contents of aspartic acid and glutamic acid were found by others for collagen VI. Most striking, however, is the glycine content of 18%, which is much lower than the one third glycine content in other collagens (Sage and Bornstein, 1982) and that we found also in a control experiment with collagen I. This relatively low glycine content was found in all cited reports as typical for collagen VI.

Immunocytological Localization of the Antigen Recognized by 3D7 in Early Embryogenesis

The temporal and spatial patterns of 3D7 immunoreactivity were analyzed by immunocytochemistry on glutaraldehyde/paraformaldehyde-fixed paraffin embedded sections of *Xenopus laevis* embryos (Fig. 3). The specificity of the 3D7 immunoreactivity was checked by using a nonreacting mouse IgM mAb, instead of 3D7 or by using the second FITC-conjugated antibody only. In these cases, no staining was found (results not shown). In the unfertilized egg, the antigen is evenly distributed in the cytoplasm (Fig. 3 *A*). Within 30 min after fertilization, however, a striking redistribution of the antigen occurs (Fig. 3 *B*). The antigen becomes localized as a broad band in the periphery of the zygote. In the cleavage stages, the antigen remains localized in the peripheral parts of the cytoplasm (Fig. 3, *C* and *D*). When more cell layers develop, this peripheral cytoplasmic localization is restricted to the peripheral cell layers (Fig. 3, *D* and *E*). The intracellular localization was also restricted to the peripheral cell layers in the blastula stage (Fig. 3, *E* and *F*). The antigen is available both intracellularly and extracellularly. Cell membrane of cleavage stage embryos are also stained and staining is observed between the individual cells (Fig. 3, *C–F, I–L*). Both the peculiar peripheral localization and the presence of the antigen between the cells gave rise to the idea that the antigen was secreted.

The most interesting localization of the antigen was found during gastrulation (Fig. 3, *G* and *I*). Staining was found in the cells lining the blastopore and in the extracellular space between the two cell layers, in the presumptive archenteron. The two involuting cell layers which contribute to the presumptive archenteron are a more animal, outer cell layer (roof) and a more vegetal, outer endodermal cell layer (*bottom*). These cell layers also contain the immunocytochemical staining (see Fig. 3, *E* and *F*) before gastrulation starts. So, the apparent relocalization of staining results, in fact, from involution of the cell layers that were stained before gastrulation started. This staining does, however, suggest a role for antigen in gastrulation (see below). No antigen was detectable between the involuting mesoderm and the overlying ectoderm, the area in which both fibronectin (Lee et al., 1984) and laminin (Darribère et al., 1987) accumulate during gastrulation.

During the neurula (Fig. 3 *H*) and also in later stages (results not shown), however, the antigen is found only in the presumptive epidermis.

In summary, the most notable facts concerning the localization of the antigen are as follows. The localization changes dramatically during the earliest embryonic stages. The antigen becomes localized after fertilization. From then, until the blastula stage, intracellular antigen is localized exclusively in the periphery of the embryo. Extracellular antigen is found between all cells in the earliest stages, but also tends to become localized at the periphery of the embryo as development proceeds. During gastrulation, the antigen is also localized in the blastopore region, inside the cells and between the cell layers that line the presumptive archenteron. Extracellular antigen decreases strongly elsewhere in the embryo at this stage.

The immunocytochemical detection of the antigen by 3D7 appeared to depend on the presence of Ca^{2+}- and Mg^{2+}-dependent cell–cell adhesion. Early cleavage stage embryos were placed in Ca^{2+}- and Mg^{2+}- free medium, which leads to diaggregation of the embryo into loose cells. The immu-

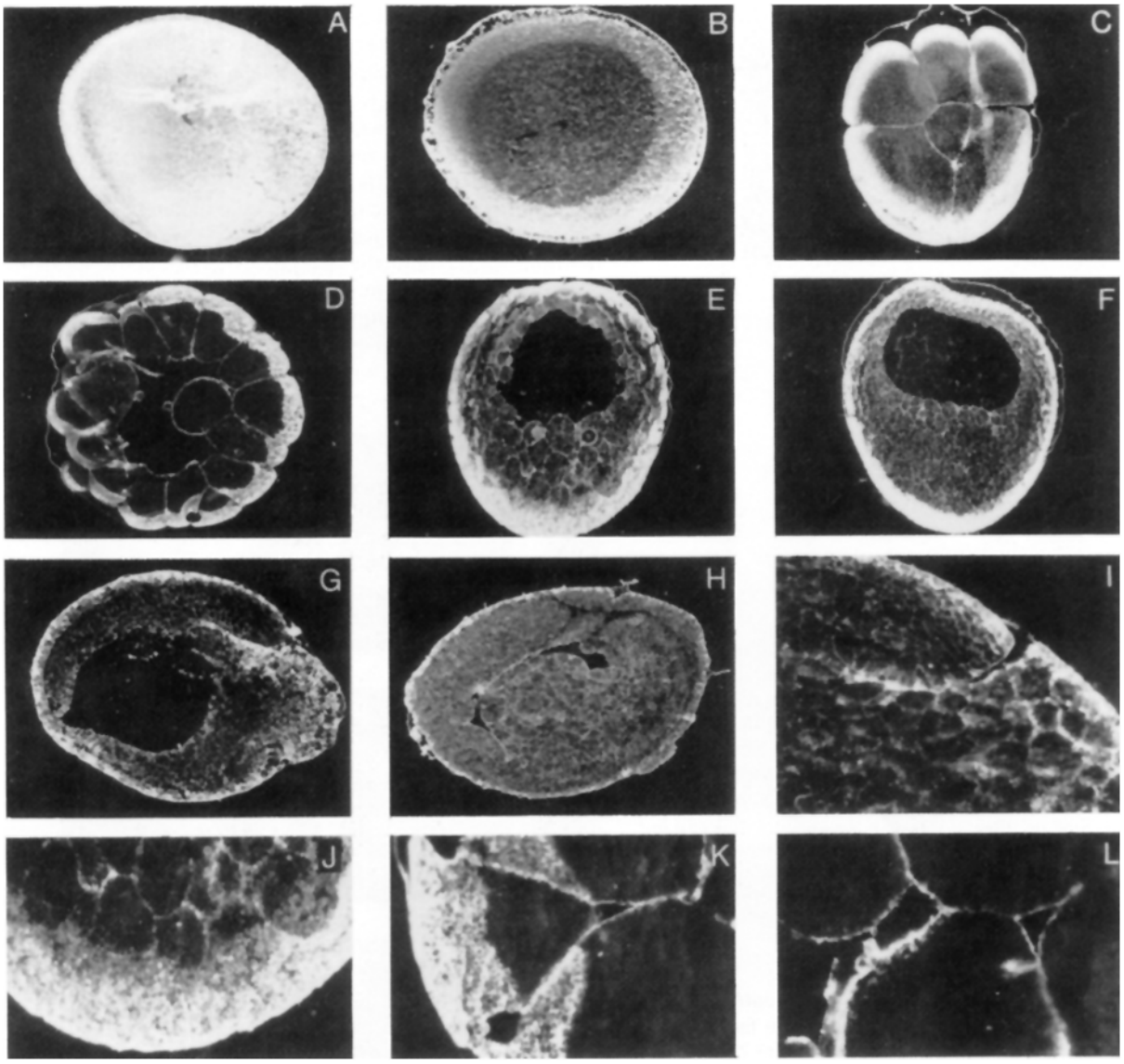

Figure 3. Distribution of the antigen recognized by 3D7 in sections of early *Xenopus laevis* embryos. 8-μm sections of glutaraldehyde/paraformaldehyde-fixed embryos embedded in paraffin were stained by indirect immunofluorescence using the 3D7 antibody and FITC-conjugated GAM IgG. (*A*) Unfertilized egg; (*B*) fertilized egg, 30 min after fertilization; (*C*) stage 4 embryo; (*D*) stage 6 embryo; (*E*) stage 7 blastula; (*F*) stage 8 blastula; (*G*) stage 10 1/2–11 gastrula; (*H*) stage 16 neurula; (*I*) blastopore region of stage 10 1/4 gastrula; (*J*) detail of *E*, stage 7 blastula; (*K*) detail of *D*, stage 6 embryo; (*L*) detail of stage 8, mid-blastula embryo.

nocytological staining by 3D7 almost completely disappeared both intra- and extracellularly (Fig. 4 *A*). When the embryos were replaced in Ca^{2+}- and Mg^{2+}-containing medium, reaggregation of the cells occurred and immunocytological staining by 3D7 reappeared (Fig. 4 *B*). Curiously, only the extracellular staining reappeared and a flocculent, specifically stained material was also found in the blastocoel (compare Fig. 4 *B* to Fig. 3, *D* and *E*). We repeated the experiment and analyzed the antigen using Western blotting. The antigen (Fig. 4, lane *1*) disappeared when the embryos were disaggregated in Ca^{2+}- and Mg^{2+}-free medium (lane *2*). When the embryo was replaced in Ca^{2+}- and Mg^{2+} containing medium, the antigen reappeared after 45 min (lane *3*), and became stronger after 2 h (lane *4*), confirming the immunocytological results.

A Possible Involvement of 3D7 Antigen in Gastrulation Movements

The presence of the antigen in and between the cell layers that line the newly formed archenteron (Fig. 3, *G* and *I*) suggests a role for the 3D7 antigen in gastrulation. We tested this idea by testing the ability of 3D7 to interfere with gastrulation. Blastula embryos from which the vitelline membrane was removed, were incubated in medium containing a low concentration (0.2 mg/ml) of 3D7 antibody (intact or cleaved to monovalent antibodies). The vitelline membrane was removed to give the antibody the opportunity to bind to the outer cell layers of the blastula embryo (see Fig. 3 *F*). In all cases, the dorsal blastoporal groove appeared normally (Fig. 5 *B*). In normal control embryos the blastoporal groove

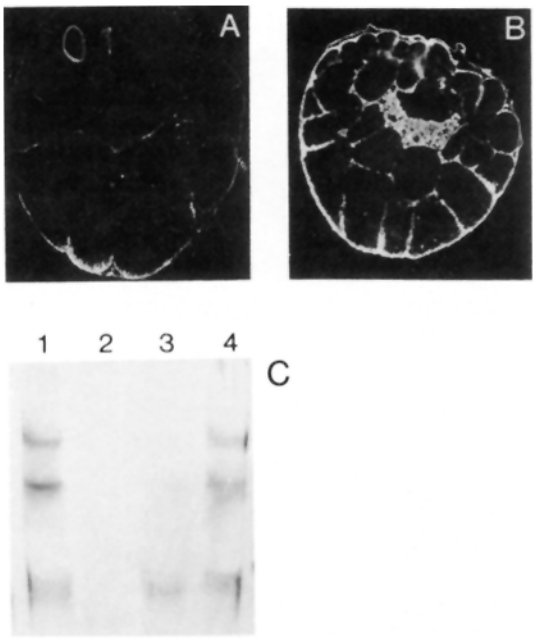

Figure 4. Disappearance of immunocytological staining after the loss of cell-cell contacts in Ca^{2+} and Mg^{2+} free medium. Stage 2 embryos were put in Ca^{2+} and Mg^{2+}-free MMR in which they disaggregate to loosely connected cells. When embryos reached stage 6 they were either fixed immediately (*A*) or after incubation in Ca^{2+} and Mg^{2+}-containing MMR for 45 min. (*B*). After fixation, the embryos were paraffin embedded and prepared for immunocytochemistry using the 3D7 antibody (*C*). Immunoblot of blastula supernatant of embryos in *A* and *B*. The 140-, 200-, and 240 kD components of the antigen from nontreated embryos are shown in lane *1*. Disaggregated embryos are shown in lane *2*. Embryos were reaggregated for 45 min (lane *3*), or 2 h (lane *4*) before analysis.

forms progressively from the dorsal to the ventral side as gastrulation proceeds, as a consequence of the fact that the cells on the ventral side also start to gastrulate (Fig. 5 *A*). This extension was blocked in embryos that were treated with 3D7 (Fig. 5, *B* and *C*). Histological sections of these 3D7-treated gastrulas revealed that the dorsal mesoderm had migrated normally (Fig. 5 *H*), to the same extent as in normal gastrula embryos (Fig. 5 *G*). This indicates that 3D7 (which is present in the medium) does not interfere with the internal involution of mesoderm during gastrulation. This treatment led in most cases (80 ± 10% in seven different batches of embryos, using 30 embryos per experiment) to exogastrulated embryos (Fig. 5, *C* and *D*). The remaining 20% of the embryos overcame the block and delayed but complete gastrulation took place. The delay in gastrulation caused a delay in development in comparison to control embryos. When control embryos had reached stage 26 (Fig. 5 *E*, Nieuwkoop and Faber, 1976), the 3D7-treated embryos had reached only stage 23 (Fig. 5 *F*). This might be a consequence of the incomplete involution of the mesoderm, due to the delaying action of 3D7. The effects of 3D7 on gastrulation were very specific. Anti-mouse EGF receptor mAbs (applied at the same concentration) had no effect, but these antibodies did not bind to *Xenopus* gastrula cells. Therefore, we also treated an antibody (called 2A9) that binds to the surface of the cells in the outer blastula and gastrula cell layers (Fig. 6, *A* and *B*). Applied in the same manner, at the same concentration, this antibody did not interfere with gastrulation, which proceeded normally (Fig. 6 *C*). In another control experiment, we injected 3D7 antibodies into the blastocoel of blastula embryos. To reach at least the same final concentration, we injected 60 nl with a five-times higher (1 mg/ml) antibody concentration than we used when the antibody was present in the culture medium. This experiment was designed in analogy with experiments with antifibronectin antibodies that block gastrulation in *Pleurodeles waltl*, when injected into the blastocoel (Boucaut et al., 1984*a*). The antifibronectin antibodies had to be injected into the blastocoel to have access to the localized fibronectin

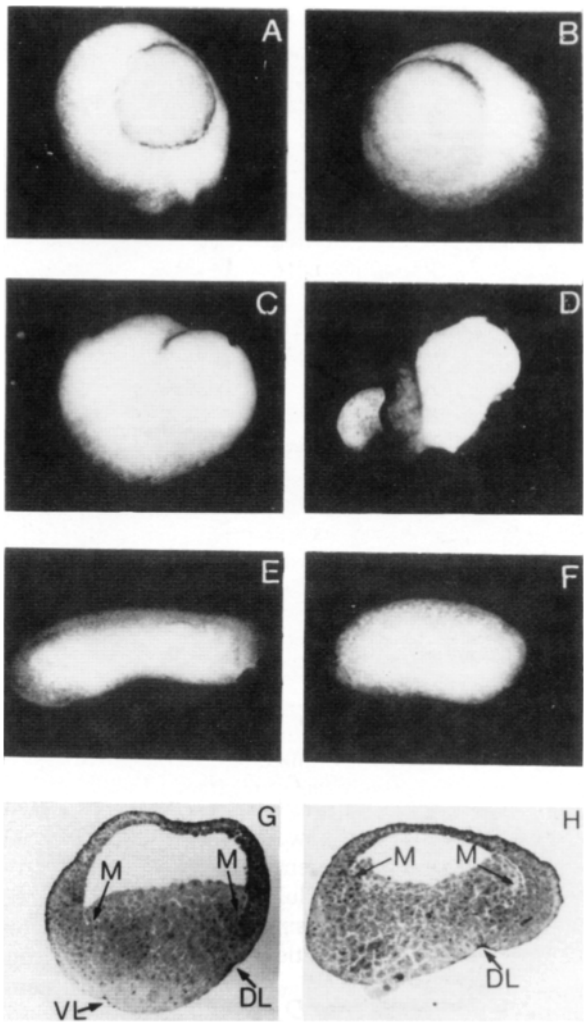

Figure 5. 3D7 antibody blocks gastrulation when present in the incubation medium. The vitelline membranes were removed manually from stage 7 blastulas and the embryos were cultured in MMR containing monovalent or intact 3D7 antibodies (0.2 mg/ml) (*B–D*, *F* and *G*) or nonsense hybridoma antibodies (0.2 mg/ml) (*A* and *E*). Photographs were taken during gastrula stages (*A–C*) or when the control (*E*) embryos reached stage 26. 3D7-treated embryos either exogastrulated (*D*) or were delayed in their development (*F*). Cross-sections of nontreated embryos (*G*) and 3D7-treated embryos (*H*) were made when embryos had reached the stage shown in *A* and *B*. The involuting mesoderm is indicated (*M*), as well as the dorsal (*DL*) and ventral (*VL*) blastopore lip.

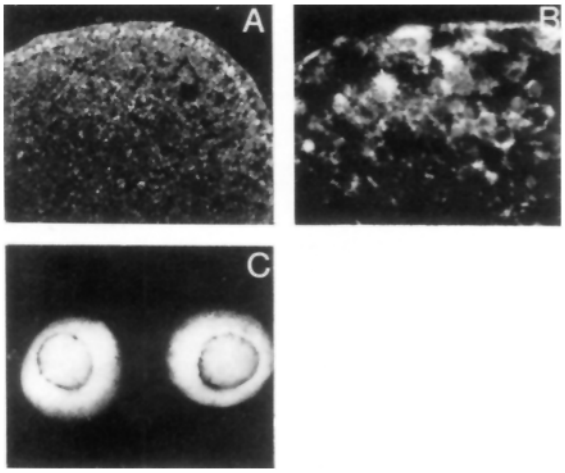

Figure 6. A cell surface-binding antibody which does not block gastrulation. An antibody (2A9) that binds to gastrula cells (*A* and *B*) was added to the culture medium at the same concentration (0.2 mg/ml) as was used for 3D7 (Fig. 6). 2A9 did not interfere with gastrulation (*C*).

between the involuting mesoderm and ectoderm (Lee et al., 1984). As the 3D7 antigen is not localized between mesoderm and ectoderm, no effect of injected 3D7 antibody was to be expected. This proved to be correct. 3D7 antibody, injected into the blastocoel, had no effect on gastrulation and the embryos developed normally. The ability of 3D7 antibodies to block gastrulation when added from outside and their failure to do so when injected into the blastocoel, correlates well with the localization of the 3D7 antigen in the newly formed archenteron and its absence between the mesoderm and ectoderm. It also correlates with the observation that the initial involution of the dorsal mesoderm is not blocked when 3D7 is present in the medium (Fig. 5, *G* and *H*).

Discussion

The purpose of this investigation was to characterize an extracellular matrix element in the earliest embryonic stages of *Xenopus laevis* development. An important structural and regulatory role for extracellular matrix elements is likely in these stages because they show extensive morphogenetic events, cell divisions and differentiation. Here, we describe a maternally expressed extracellular matrix element that we found very similar to mammalian collagen type VI and that is possibly involved in gastrulation movements.

The Antigen Recognized by 3D7 Is Very Similar to Collagen Type VI

Our results indicate that the antigen recognized by 3D7 is collagen type VI. This conclusion is based on the following observations. (*a*) The collagenous character is established by the presence of both OH-proline and OH-lysine in the purified antigen. (*b*) The glycine content of the purified antigen (purity assessed by silver staining of a SDS-PAGE gel) is higher than in noncollagenous proteins but considerably lower than in collagen I–V (Sage and Bornstein, 1982). This relatively low glycine content for collagens has been found to be typical for collagen VI and is probably due to noncollagenous sequences in collagen VI (Carter, 1982*a*; Hessle and Engvall, 1984; Trüeb and Bornstein, 1984; Trüeb and Winterhalter, 1986; Timpl and Engel, 1987). (*c*) The antigen is degraded by bacterial collagenases. The insensitivity of particularly the 40–47-kD components under nonreducing conditions and their sensitivity under reducing conditions has been found by others (Heller-Harrison and Carter, 1984; Trüeb and Bornstein, 1984; Trüeb and Winterhalter, 1986) and is specific for collagen VI. The insensitivity of the 40–47-kD cluster to pepsin digestion makes it likely that this cluster consists of the so-called short-chain collagen, which is considered to be a breakdown product, of the native higher molecular weight components generated by isolation procedures. (*d*) The molecular weights, especially of the higher molecular weight components, fit those of native collagen VI, as found by several groups. Both the 200- and 240-kD components have been found to be secreted by fibroblasts (Hessle and Engvall, 1984; Trüeb and Bornstein, 1984; Trüeb and Winterhalter, 1986). The 240-kD component is considered to be the precursor of the 200-kD component (Trüeb and Winterhalter, 1986).

Possible Functions of Collagen VI

So far, no role has been established for collagen VI in early vertebrate embryogenesis. Although an increasing amount has been discovered about the structure and processing of this type of collagen (Burgeson, 1988; Chu et al., 1988), its function remains an enigma (Engel et al., 1985; Timpl and Engel, 1987; Burgeson, 1988). It has been suggested that it is involved in cell–cell adhesion in human fibroblasts (Carter, 1982*a*) and its biosynthesis is almost completely blocked in transformed fibroblasts, which have altered adhesive properties (Carter, 1982*b*; Truëb et al., 1985). Further, fibroblasts from a patient with Cutis Laxa (a disease that is characterized by a loss of skin elasticity) were shown to have decreased collagen VI synthesis and secretion, suggesting a role for collagen VI in mechanical flexibility of tissues (Crawford et al., 1985). Both changes in cell–cell adhesion and extensive morphogenetic movements requiring a high flexibility of the tissues take place during the early embryonic development of *Xenopus laevis*. The presence of the 3D7 antigen, both intracellularly and extracellularly, during the earliest embryonic stages of *Xenopus laevis*, suggest that it plays more than one role during early embryonic development. Our results suggests at least a possible role in gastrulation movements. Its localization, during gastrulation, in the developing archenteron, makes such a role feasible. The ability of relatively low concentrations of Fab elements of the 3D7 antibody to block gastrulation also supports this idea. These effects were specific for 3D7, since another antibody, which binds to the outer cell layers in gastrula embryos, did not interfere with gastrulation. That a gastrulation block only occurs when 3D7 is presented outside the embryo is explained by the localization of collagen VI in the developing archenteron. Injection of high concentrations of 3D7 antibodies into the blastocoel had no effect on gastrulation. This is not surprising, as there is no collagen VI between the mesodermal and ectodermal cell layers. Injection of 3D7 into the blastocoel was performed in analogy with the experiments with anti-fibronectin antibodies (Boucaut et al., 1984*a*). These experiments, however, were performed in

Pleurodeles waltl, in which they blocked gastrulation. It is dangerous to extrapolate these results to *Xenopus laevis*.

The way in which the 3D7 antibody may interfere with the function of collagen VI is unclear. The anti-fibronectin antibody appears to interfere with the binding of fibronectin to its receptor (Boucaut et al., 1984*b*). It is not known whether collagen VI has a specific receptor. Whatever the mechanism via which 3D7 acts, it is clear that the roles of fibronectin and collagen VI in gastrulation are different. Gastrulation in *Xenopus laevis*, is very complicated as the marginal zone exists of two cell layers; a superficial layer of prospective endoderm and a deep region of prospective mesoderm. Gastrulation starts in the inner cell layer when the inner marginal zone mesoderm rolls inwards. This happens before any invagination of the archenteron has taken place. When the dorsal blastoporal groove appears, the internal blastopore lip has already extended from the dorsal to the lateral and even to some extent to the ventral side (Nieuwkoop and Faber, 1967; Gerhart and Keller, 1986). Anti-fibronectin antibodies (in *Pleurodeles waltl*) interfere with the initial, internal process, and are therefore very effective in blocking gastrulation completely in this species. The 3D7 antibody interferes with the later, more external process. This may well be the reason why 3D7 is less efficient in blocking gastrulation. It can act only after the internal aspects of gastrulation have started.

We are well aware that the difficulty with using gastrulation or exogastrulation as an assay in amphibian development is that almost any substance, present at a high enough concentration in the medium will cause exogastrulation. We think, however, that the (initial) experiments reported here, indicate a possible role for the 3D7 antigen in gastrulation movements. The 3D7 antigen is first of all present in and between the cell layers that line the developing archenteron and the fact that the 3D7 antibody is only able to interfere with gastrulation when applied externally, is in full agreement with this localization. An important control experiment, in which an antibody that also binds to gastrula cells did not interfere with the gastrulation process, underlines the specificity of the 3D7 effects.

The differential effects of antibodies against different extracellular matrix elements like fibronectin or the 3D7 antigen here, on gastrulation, show that it may be fruitful to use mAbs as tools to unravel complex events such as gastrulation movements.

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