Polar asymmetry in the organization of the cortical cytokeratin system of *Xenopus laevis* oocytes and embryos

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This work is dedicated to the memory of Richard C. Parker (1952–1986)

Summary

We have used whole-mount immunofluorescence microscopy of late-stage *Xenopus laevis* oocytes and early embryos to examine the organization of their cortical cytokeratin systems. In both mature oocytes and early embryos, there is a distinct animal–vegetal polarity in cytokeratin organization. In mature (stage-VI) oocytes, the cytokeratin filaments of the vegetal region form a unique, almost geodesic network; in the animal region, cytokeratin organization appears much more variable and irregular. In unfertilized, postgerminal vesicle breakdown eggs, the cortical cytokeratin system is disorganized throughout both animal and vegetal hemispheres. After fertilization, cytokeratin organization reappears first in a punctate pattern that is transformed into an array of oriented filaments. These cytokeratin filaments appear first in the vegetal hemisphere and are initially thin. Subsequently, they form bundles that grow thicker through the period of first to second cleavage, at which point large cytokeratin filament bundles form a loose, fishnet-like system that encompasses the vegetal portion of each blastomere. In the animal region, cytokeratin filaments do not appear to form large fibre networks, but rather appear to be organized into a system of fine filaments. The animal–vegetal polarity in cytokeratin organization persists until early blastula (stage 5); in later-stage embryos, both animal and vegetal blastomeres possess qualitatively similar cytokeratin filament systems. The entire process of cytokeratin reorganization in the egg is initiated by prick activation. These observations indicate that the cortical cytoskeleton of *Xenopus* oocytes and early embryos is both dynamic and asymmetric.

Key words: *Xenopus*, oocytes, embryos, intermediate filaments, cytoskeletal polarity.

Introduction

The *Xenopus laevis* oocyte is a highly organized cell (see Gerhart, 1980 for review). Like all higher eukaryotic cells, it possesses a discrete region associated with the inner surface of the plasma membrane, the cortex, and a deeper inner cytoplasmic mass, the endoplasm (Elison, 1980). It also possesses a dramatic axis of radial symmetry, the animal–vegetal polar axis (see Nieuwkoop, 1977). This axis is defined by the position of the oocyte nucleus and the distribution of cortical pigment granules, cytoplasm, yolk platelets and germinal granule material. The polar asymmetry of the oocyte appears to be defined early in oogenesis (Nieuwkoop, 1977; Gerhart, 1980) and is first macroscopically visible as the axis defined by the mitochondrial cloud (Balbiani body) and the germinal vesicle.

In response to hormones, the oocyte matures into the egg. During this process, the oocyte nucleus, the germinal vesicle, breaks down and releases its contents into the cytoplasm; the meiotic spindle moves toward the egg surface, where it produces the animal pole, the site of polar body extrusion and a region of the animal hemisphere free of pigment. This meiotic spindle is arrested in metaphase II, awaiting fertilization by the sperm (Brachet, Hanoqc & Van Gansen, 1970; Gerhart, 1980). Productive fertilization generally occurs in the animal hemisphere and the introduction of the sperm nucleus/centriole complex initiates a series of changes in both the egg cortex and the endoplasm (Brachet, 1977; Palacek, Ubbels & Rzhak, 1978; Elison, 1980, 1983, 1985; Stewart-Savage & Grey, 1982; Ubbels, Har, Koster & Kirschner, 1983 and references therein). These changes include the cortical granule reaction, the
contraction and subsequent relaxation of the cortex, the formation of the fertilization envelope, the gravity-driven rotation of the egg within the perivitelline space, the formation of a sperm aster, the resumption of meiosis, and the migration and fusion of male and female pronuclei. Many of these changes also occur in response to mechanical, electrical or ionophore-induced egg activation, indicating that they are independent of the male pronucleus. Together, maternally preprogrammed and sperm-dependent events culminate in the reorganization of the egg cytoplasm and the apparent rotation of the egg cortex with respect to the endoplasm (Ancel & Vitemberger, 1948; Vincent, Oster & Gerhart, 1986). The end result of this cascade of events is the breaking of the egg's original radial symmetry and the formation of a second axis which will eventually result in the formation of the dorsal–ventral axis of the embryo (Gerhart & Keller, 1986).

The rotation of egg cortex with respect to the endoplasm implies that the two regions are physically distinct. It has been known for some time that the oocyte/egg cortex has a structural integrity and can be mechanically isolated away from the endoplasmic mass (see Holtfreter, 1943; Franke et al. 1976; Elison, 1983; Gall, Picheral & Gounon, 1983) as a discrete unit. The cortex contains large numbers of microfilaments, cytoteratin-type intermediate filaments, microtubules, cortical granules, pigment granules and various membranous organelles (Franke et al. 1976; Campanella & Andreuccetti, 1977; Elison, 1980; Gall et al. 1983; Franz et al. 1983; Godsave, Wylie, Lane & Anderton, 1984).

While it is clear that certain components of the cortex, such as pigment granules, are organized in an asymmetric fashion, the structural basis of this asymmetry remains unknown. Previous studies on cytoskeletal components of the cortex have relied primarily on sections through the oocyte and the egg (Franz et al. 1983; Godsave et al. 1984). These workers failed to note any significant polar asymmetry in cortical cytoskeletal organization, although Godsave et al. (1984) did find a difference in the distribution of endodermal cytokeratin filaments between animal and vegetal regions. As a prelude to studies on the function of intermediate filaments within Xenopus embryos, we have developed a monoclonal antibody directed against a 56 x 10^3 (M_r) neutral/basic cytokeratin of Xenopus oocytes and early embryos together with a whole-mount labelling procedure that enables us to visualize the detailed organization of the cortical cytokeratin system of oocytes, eggs and embryos. Using this method, we have discovered a polar asymmetry in the organization of the cortical system in both oocytes and early embryos.

### Materials and methods

#### Xenopus care and manipulation

Adult male and female *Xenopus laevis* were obtained from John Gerhart (UC Berkeley) and maintained in specially constructed tanks in 10 mM-salt solution. Animals were fed every day with Purina Trout Chow and their water was changed after each feeding. After handling, animals were maintained for 2 to 5 days in 50 mM-NaCl solution in order to promote the healing of any superficial trauma they may have experienced.

Testes were obtained from males injected two days previously with 50 i.u. pregnant mare's serum (PMS) (Calbiochem). These animals were killed with benzocaine and their testes were dissected and stored in 10% fetal calf serum, x1 complete Ringer's (Ringer's: 0.1M-NaCl, 1.8 mM-KCl, 2 mM-CaCl_2, 1 mM-MgCl_2, 5 mM-Hepes pH 7.8) and 50 µg/ml gentamycin at 4°C until use, typically within one week of dissection. To produce eggs, mature females were injected with 50 i.u. PMS; 8 to 12 h later, they were injected with 200 to 600 i.u. human chorionic gonadotropin (Sigma) and held at 15°C until the next morning. Mature eggs were stripped and fertilized by exposure to masked tetras testes and held for 5 to 10 min in 33% Ringer's solution. Eggs were then dejellied by exposure to 2% cysteine, pH 7.9 for 5 min. After removal of the jelly coat, the fertilized eggs were washed three times in 33% Ringer's and maintained in 20% Ringer's supplemented with 50 µg/ml gentamycin throughout the remainder of the experiment. For 'prick-activation', mature eggs were dejellied and transferred into a solution of 5% Ficol 400 in 20% Ringer's; they were activated by prickings with a clean glass needle. Eggs were held for prickings on a plate of 2% Agarose in which 1.5 to 2 mm depressions had been made.

To obtain mature oocytes, recently spawned females were anaesthetized with benzocaine. A small incision was made through skin and body wall muscle; pieces of ovary were teased out with a cotton-tipped stick and cut away with scissors. The remaining ovarian tissue was returned to the body cavity and the incisions closed with three or four sutures in the body wall muscle and then in the skin. Oocytes were prepared from ovarian tissue by dissociation with 2.5% collagenase in Ca^2+/-, Mg^2+/--free Ringer's overnight at 16°C with constant gentle rocking. The dissociated oocytes were washed three times in complete Ringer's and then fixed and stored in −20°C methanol.

#### Monoclonal antibody production and characterization

The Xenopus laevis-derived kidney epithelial cell line A6 (A6) (obtained from American Type Cultures Collection) was grown in 85% Leibowit-L15 media supplemented with newborn calf sera and gentamycin. Cells were grown in closed flasks or on sterile glass coverslips in Petri dishes in a 25°C incubator. To prepare cell residues, flasks of confluent cells were washed three times with cold phosphate-buffered saline containing 10 mM-EGTA and 0.5 mM-PMSF (PBS'); the cells were then extracted with cold PBS' containing 0.5% NP-40 until the cells were released from the tissue culture surface. The extracted cells were collected by centrifugation and extracted again with PBS'/NP-40; after
centrifugation, the cell extracts were washed twice more
with PBS+. After the final wash, the cell extract was
resuspended in either PBS+ or in Laemmli (1970) sample
buffer.

For SDS–PAGE analysis, samples solubilized in
Laemmli buffer were sonicated and heated at 90°C prior to
loading on the gel. After electrophoresis, proteins were
electrophoretically transferred to nitrocellulose paper and
excess protein-binding sites were blocked with 2 % low-fat
dried milk dissolved in Tris-buffered saline (TBS). For
Western analysis, spent tissue culture supernatants were
used; in each case, 20 % newborn-calf serum was added (in
addition to the 10 % fetal calf serum used to grow the
hybridomas originally) and the strips were incubated in the
primary antibody overnight at 4°C. Strips were washed
twice with TBS made 1 M in NaCl and then three times
in TBS; each wash was for 5 min on a rocking platform.
The strips were then incubated in affinity-purified goat
anti-mouse immunoglobulin conjugated to horse radish
peroxidase (antiMIg-PO) (Biorad) diluted 1:3000 in TBS
supplemented with 20 % newborn-calf serum. After incu-
bation at room temperature for 1–2 h, the strips were
washed as before and developed using 4-chloro-1-naphthol
and H2O2 (Hawkes, Niday & Gordon, 1982). After 10 min,
the strips were washed 5 to 10 min in distilled water and
then dried.

For immunization of mice, cell extracts in PBS7 were
emulsified according to manufacturer's direction in RIBI
adjuvant; 1-month-old, female Balb/c mice were immu-
nized by intraperitoneal injection with 0.2 ml (0.05 mg) cell
residue protein; mice received booster immunizations every
two weeks for 2 to 3 months. Prior to the use of a mouse to
prepare monoclonal antibodies, the animal received a final
intravenous injection (0-05 ml) of antigen. Protein concen-
trations were measured using the Biorad Coomassie bril-
liant blue colorimetric assay.

Antisera responses were monitored by immunofluores-
ence microscopy on methanol-fixed A6 cells; immuno-
fluorescence microscopy of cultured cells was carried out as
described previously (Klymkowsky, Miller & Lane, 1983).
A mouse with a strong (1:10000) immune titre against the
cytokeratin system of A6 cells was chosen, killed by cervical
dislocation and its spleen dissected. Spleen cells were
prepared and fused with P3 myeloma cells according to
standard procedures (Hudson & Hay, 1979). All hybrid-
oms were grown in RPMI-1640 supplemented with 10 %
heat-inactivated fetal calf serum, nonessential amino acids,
50 µM-2-mercaptoethanol and 50 µg ml–1 gentamycin. The
monoclonal antibody 1h5 (IgG) was obtained by repeated
cloning by limited dilution.

During the characterization of 1h5, we also examined the
specificities of the monoclonal antibody antiIFA (IgG)
(Pruss et al. 1981) (obtained from American Type Culture
Collection) which reacts with most, if not all, intermediate
filament subunit proteins; the monoclonal antibody AE1
(IgG) (Sun et al. 1985) (supplied by T. T. Sun – NYU
Medical School) which reacts specifically with the acidic
cytokeratins of mammalian epithelial tissues; Id7 (IgM), a
monoclonal antibody specific for nuclear lamins (Klym-
kowsky, unpublished observations); 14h7 (IgG), a mono-
clonal antibody that appears specific for Xenopus vimentin
(unpublished observations – see Results); RV202, a mono-
clonal anti-vimentin antibody supplied by F. Ramaekers
(U. Nijmegen, The Netherlands); and E7 (antitubulin)
(IgG), a monoclonal anti-beta tubulin-specific antibody
generated by S. Carroll and M. McCutcheon (UC
Boulder). All of these monoclonal antibodies were used for
both immunofluorescence microscopy and Western immu-
noblot analysis as spent tissue-culture supernatants.

Immunocytochemistry

Immunofluorescence microscopy of A6 cells was carried
out as described previously for cultured mammalian cells
(Klymkowsky et al. 1983). Xenopus oocytes, dejellied eggs
and embryos were fixed in –20°C methanol overnight.
After fixation, the methanol was removed and the eggs were
extracted with 60 mm-Pipes, 25 mm-Hepes, 10 mm-
EGTA, 3 mm-MgCl2, 1 m-hexylene glycol and 0.15 % Tri-
ton X-100 pH 6.9 for 15–30 min at room temperature with
constant rocking; similar results were obtained when ex-
traction was carried out using PBS supplemented with 1 m-
hexylene glycol and 0.15 % Triton X-100. During the
transfer from methanol to extraction buffer, the vitelline
membrane often cracked off the egg. After extraction, the
buffer was removed and the eggs were incubated in antibody
overnight at 4°C. The eggs were then washed five
times for 5 min each in PBS and then incubated for 2 h at
room temperature in fluorescein-conjugated, affinity-puri-
fied goat anti-mouse IgG+IgM+IgA antibody (antiMIg-Fl)
(Cappel) diluted 1:100 in PBS/0.15 % sodium azide. The eggs
were then washed as before and placed in the centre of a 0.8 mm deep depression slide; excess liquid was removed and gelvatol (Klymkowsky
et al. 1983) was placed over and around the eggs. A clean glass
coverslip was placed over the eggs and gentle pressure was
used to remove excess gelvatol and to deform the eggs
slightly.

The 1h5-epitope is sensitive to aldehyde fixation, but will
withstand mild fixation (1 % formaldehyde in PBS for
30 min at room temperature) without a drastic loss of
reactivity. One batch of eggs was examined using this
fixation protocol (followed by cold methanol) and it gave
results identical to those obtained using methanol fixation
alone.

For cryosectioning, methanol-fixed oocytes/embryos
were incubated in Tissue-Tek mounting media for
20–30 min at room temperature; the specimen was then
placed on a cryostat chuck and frozen at –30°C. 10 µm
sections were cut on a SLEE cryostat (generously loaned to
us by Dr W. Wood, UC Boulder) and were picked up onto
subbed coverslips. Sections were stained with antibody
solution for 3 h at room temperature, washed six times with
PBS, incubated in antiMIg-Fl for 3 h, washed and mounted
in gelvatol.

Microscopy was carried out on a Zeiss IM35 microscope
equipped with phase and Nomarski optics using either a
63X neofluor (loaned to us by Dr J. Van Blerkom, UC
Boulder), a ×25 plan-neofluor or a ×63 planapochromatic
lens. Photographs were taken onto either Kodak Tri-X or
Ektapan (400 ASA) film developed to give an effective film
speed of 800 ASA. In some cases, photographic prints were
made directly from colour slides. Nonspecific labelling was
not observed when antiMg-Fl was used alone; in addition, the antibodies AE1, antiIF and 14h7 (all IgG) showed no apparent labelling of whole-mount, 2-cell embryos.

Results

Monoclonal antibody characterization

Franz et al. (1983) reported that the Xenopus-derived cell line A6 expresses the same three cytokeratin proteins as are found in the mature oocyte. Detergent-insoluble residues of A6 cells are highly enriched in these cytokeratin proteins and such cell residues were used to immunize mice for the production of monoclonal antibodies (see Methods). One of these antibodies, ih5, displayed a particularly strong and specific labelling of the cytokeratin filaments of methanol-fixed A6 cells (Fig. 1A). ih5 labelled the cytokeratin filaments of mitotic cells more intensely than interphase cells (not shown), a behaviour that has been described in the case of other anti-cytokeratin antibodies in mammalian cells (Franke et al. 1983). On intracellular injection into A6 cells, concentrated 1h5 (10 mg ml⁻¹) antibody labelled only cytokeratin filaments and produced a specific disruption of cytokeratin organization within 1 h (unpublished observations) in a manner reminiscent of the effect of other monoclonal anti-cytokeratin antibodies on the cytokeratin filaments of mammalian cells (Klymkowsky, 1982; Klymkowsky et al. 1983).

Western immunoblot analysis of A6 cell residues (Fig. 1B) and 4-cell Xenopus embryos (not shown) indicated that 1h5 reacts specifically with a 56 000 Mᵣ (56K) cytokeratin protein. The specificity of antibody labelling under these conditions was monitored by comparing the labelling of 1h5 with that of the monoclonal antibodies antiIF, AE1, 14h7 and 1d7 (see Methods). The 56K, 1h5-labelled cytokeratin protein was strongly labelled by antiIF, which reacts with most, if not all, intermediate filament subunit proteins (Pruss et al. 1981) (Fig. 1B). AE1, which reacts specifically with the acidic cytokeratins of mammalian cells (Sun et al. 1985), reacted most.

Fig. 1. Analysis of 1h5 specificity in Xenopus A6 cells. (A) A micrograph, taken in fluorescein-optics of methanol-fixed, 1h5-labelled A6 cells demonstrates that 1h5 labels cytokeratin filaments (Bar, 10 μm). (B) Detergent-insoluble cell residue proteins of Xenopus A6 cells were separated by SDS–polyacrylamide gel electrophoresis and either stained with Coomassie brilliant blue (lane marked 'cell residue') or were electrophoretically transferred to nitrocellulose paper and probed with the monoclonal antibodies antiIF, 1h5, 14h7, AE1 or 1d7. Relative molecular mass marker positions (×10⁻³) are noted on the left hand side of the figure; the positions of major protein bands have been marked across the figure.
protein has been identified as vimentin based on whether it is the 55K cytoskeletal protein described in cell residues. It remains unclear whether this protein is a proteolytic fragment of the 56K cytokeratin or whether it is the 55K cytoskeletal protein described in passing by Franz et al. (1983). This 55K cytоссkeletal protein has been identified as vimentin based on results obtained with the monoclonal antibodies RV202 and 14h7. Both 14h7 and RV202 label a second intermediate filament system in A6 cells, distinct from the cytokeratin filament system (not shown); in western blot analysis, 14h7 reacted preferentially with the 55K cell residue protein (Fig. 1B). The presence of distinct vimentin and cytokeratin filament systems is common in cultured mammalian epithelial cells (see Traub, 1985 for review); the two intermediate filament systems can be clearly separated from one another by treating cells with nocodazole, which causes the collapse of vimentin filament organization without significant effects on cytokeratin organization (Osborn, Franke & Weber, 1980). In nocodazole-treated cells, RV202 and 14h7 both labelled a system of collapsed filaments, whereas 1h5 labelled only the extended cytokeratin filament system (not shown).

Based on these results, it appears that 1h5 specifically labels the 56K cytokeratin of A6 cells. This cytokeratin has the properties of, and may be identical with, the product of the gene for the 56K embryonic cytokeratin cloned by Franz & Franke (1986). Peptide map (Franz et al. 1983) and sequence analysis (Franz & Franke, 1986) indicate that the 56K embryonic cytokeratin is a simple epithelial cytokeratin analogous to the cytokeratin no. 8 of mammals. This is the first intermediate filament protein to be expressed during the course of mammalian embryogenesis (see Franz et al. 1983).

Whole-mount immunofluorescence microscopy

Previous studies (Franz et al. 1983; Godsave et al. 1984) used cryosectioning to localize cytokeratin proteins within the Xenopus ovary, oocyte, egg and early embryo. The cytokeratin system of the egg and early embryo appears to be confined to the cell cortex; in oocytes, cytokeratin proteins are enriched in the cortex, but are also found in the endoplasm (Franz et al. 1983; Godsave et al. 1983; Polson & Klymkowsky, unpublished observations). As a prelude to intraembryonic injection studies, we set out to determine the details of cortical cytokeratin organization within the mature oocyte and early embryo; since our goal was to monitor the effects of intraembryonically injected antibody on the fine details of cytokeratin organization, we chose a whole-mount method of staining, analogous to that which has been applied to visualizing cytokeratin proteins in Drosophila embryos (Mitchison & Sedat, 1983; Karr & Alberts, 1986). Our method involved fixing oocytes or dejellied eggs with cold methanol followed by extraction and antibody-labelling (see Methods). Gentle deformation with a coverslip of the egg mounted on a glass depression slide allowed large areas of the cortex (see Fig. 6B, D as examples) to be examined at high magnification at a resolution comparable to that obtained when examining cultured cells (compare Fig. 1 with Figs 2–6). The method enabled us to visualize fine details of cortical cytokeratin organization not readily apparent in sectioned material.

In describing the organization of cytokeratins in oocytes, eggs and early embryos, we have combined observations from three different oocyte isolations (different females) and over 30 different egg batches. In every case, the pattern of cytokeratin staining was very similar within cells derived from any one female; however, there were some variations in cytokeratin organization between batches of eggs. The cause of these variations is unclear, although it is obvious from gross observation that eggs derived from different females differ in the density and extent of their animal pole pigmentation and in their ‘softness’ (see Elison, 1983). In this work, only egg batches that produced a high percentage (over 70%) of viable embryos have been included and where significant variation between egg batches was observed these variations have been described.

Cytokeratin filaments in mature oocytes and unfertilized eggs

The largest (stage-VI) oocytes are characterized by a dense pigment granule cap in the animal region and a lighter pigment cap in the vegetal region; between the two is an equatorial zone with very little pigment (Dumont, 1972). In the vegetal region of these large oocytes, 1h5 labelled a fine network of cortical cytokeratin filaments (Fig. 2D) characterized by distinct vertices and fine interconnecting fibrils. These cytokeratin filaments were noticeably thinner than those found in A6 cells (compare Figs 1A, 2– both at the same magnification). In regions, the vegetal cytokeratin network possessed an almost geodesic quality (Fig. 2E). This type of cytokeratin organization extended throughout the vegetal region of the oocyte and through the pigment-free equatorial zone, although, in the equatorial zone it was often difficult to visualize due to the unshielded autofluorescence of the underlying yolk platelets (not shown).

The organization of cytokeratins in the animal hemisphere was much less regular than that observed...
Fig. 2. Cytokeratin organization in the mature oocyte. The labelling of cytokeratin filaments by 1h5 in the animal region of mature oocytes varied dramatically from barely detectable (A, specific staining marked by white arrows; grey regions are areas of yolk autofluorescence visible through pigment cap); through robust, but apparently disorganized (B); to clear systems of interconnected filaments (C). In the vegetal hemisphere, the organization of cytokeratin filaments is much more regular (D), consisting of thin fibres that interconnect distinct vertices. This type of cytokeratin organization is characteristic of the entire vegetal region of mature oocytes; in some regions it can approach a geometric precision (E). Arrows in part E mark vertices of the cytokeratin lattice. The bar in part E marks 10μm for all parts.
in the vegetal hemisphere. Animal pole cytokeratin organization ranged from the barely detectable, to the robust but disorganized, to that approaching the degree of organization found in the vegetal hemisphere (Fig. 2A–C). The intensity of cytokeratin labelling bore no obvious relation to the density of the animal pole pigment cap; in fact, the presence of pigment appeared to act to shield out yolk autofluorescence and made the Ih5-labelled, animal region cytokeratin filaments stand out more dramatically than the cytokeratin filament system of the vegetal region. Preliminary studies of earlier stage oocytes indicate that the geometric organization of the vegetal cytokeratin system of the mature oocytes appears relatively late in oocyte maturation.

In the majority of the mature eggs examined we found what appeared to be specific cytokeratin staining that consisted of irregular and occasionally punctate aggregates (Fig. 3B); occasionally, we found short cytokeratin filaments (Fig. 3A).

Fertilized eggs, 2- and 4-cell embryos

The earliest fertilized eggs examined in this study were fixed 15 min after fertilization, timed from the addition of macerated testis. By this time, the cortical contraction of the animal pole pigment cap was complete, the pigment cap had begun to relax back to its prefertilization position (Stewart-Savage & Grey, 1982) and the eggs had reoriented themselves within the vitelline envelope in response to gravity. In all egg batches examined, the Ih5-labelled cytokeratin system went through the same progression of changing organization; however, in some egg batches, there existed a pattern of punctate Ih5-cytokeratin labelling prior to fertilization. Examination at 15 min postfertilization revealed a fine punctate pattern of Ih5-labelling throughout the vegetal hemisphere (Fig. 3C); generally no labelling was found in the animal region (not shown). This pattern of punctate cytokeratin labelling first became more pronounced (Fig. 3D) and then was transformed into a fibrous system (Fig. 3E) between 30 and 60 min postfertilization. This punctate-to-fibrous transformation generally began in the vegetal pole region and only later embraced regions closer to the animal pole. At the time of their first appearance, there was often a clear directionality to the system of cytokeratin filaments (Fig. 3E). It is thought that the cortex and inner cytoplasmic mass of the fertilized Xenopus egg rotate with respect to one another during roughly this period of the first cell cycle (Ancel & Vitemberger, 1948; Vincent et al. 1986), and the orientation of cytokeratin filaments may reflect the direction of this rotation.

By first cleavage, the vegetal hemisphere cytokeratin filaments had begun to form large bundles (Fig. 4A), which appear similar to classically described tonofilaments and the cytokeratin filament bundles found in A6 cells (see Fig. 1). By the time of second (Fig. 4B) or third cleavage (Fig. 4D), the vegetal cytokeratin fibre system has fully matured and is quite regular in its overall organization. There was little, if any, directionality to the cytokeratin filament system by this stage (Fig. 4D). This type of vegetal cytokeratin organization persisted into late-blastula-stage embryos (Fig. 5E).

In 4- to 16-cell embryos, the vegetal system of cytokeratin filament bundles remains associated exclusively with the cortex (Fig. 4C) as determined by cryosection microscopy. The cortical cytokeratin system did not appear to descend into the embryonic interior along cleavage furrows (not shown); in experiments where cytokinesis was inhibited by treatment with cytochalasin (Bluemink, 1971), the cytokeratin system was largely excluded from the area of new membrane that forms after the regression of the abortive cleavage furrow (unpublished data).

The organization of cytokeratin filaments in the animal region also underwent a series of concentration changes during the period from fertilization to the end of second cleavage. The initially punctate distribution of cytokeratin in the animal region was transformed into distinct filaments; generally these filaments were quite fine, although occasionally thicker filament bundles were observed. In egg batches where thicker cytokeratin filaments were found in the animal hemisphere at 60 min postfertilization, they had largely disappeared by 120 min postfertilization (4- to 8-cell stage). By this time, the animal region cytokeratin system consisted of a system of apparently fragmented cytokeratin filaments (Fig. 5A). The contrast between animal (Fig. 5A) and vegetal (Fig. 5B) cytokeratin organization was particularly striking in early (prior to stage 5) (early blastula) embryos. It is not clear whether the apparent fragmentation of the animal region cytokeratin system is due to obstruction by pigment granules. However, we have found interconnected cytokeratin filament networks in the animal hemisphere of experimentally manipulated eggs (M. W. Klymkowsky & L. A. Maynell, unpublished observations), which indicates that if an interconnected cytokeratin filament system did exist within the animal region of normal embryos, we would have been able to visualize it.

The organization of cytokeratin filaments in the animal hemisphere at these early stages is distinctly different from that found in the vegetal hemisphere (Fig. 5A–C). Two kinds of animal–vegetal pole gradients in cytokeratin organization were found. In some batches of embryos, there was an apparent reorganization of the cytokeratin system into an
Fig. 3. Cytokeratin organization in eggs and early postfertilization embryos. In some eggs, 1h5 labelling consisted of scattered filament fragments (A, negative image; white arrows point to 1h5-labelled fibres); more often (B, positive image) 1h5 labelled amorphous masses and irregular aggregates in the cortex. The following series of micrographs were taken from a batch of eggs in which the unfertilized eggs showed no punctate 1h5-labelling. By 15 min after fertilization, 1h5-labelling appeared as a pattern of small spots (C, positive image) which grew larger and more irregular (D, positive image – 30 min postfertilization) and were eventually transformed into a fibrous array (E, positive image – 60 min postfertilization), with an apparent overall directionality (marked by white arrow). Bar in part E marks 10 μm for all parts.
aggregated configuration within the equatorial zone of the embryo (Fig. 5C). In other batches of embryos, the transition from vegetal- to animal-type cytokeratin organization was more uniform, with the vegetal network of cytokeratin fibres breaking down directly into the irregular cytokeratin filaments of the animal hemisphere without any apparent distinct transition zone structure (shown schematically in Fig. 7).

By stage 6 to 7, the cytokeratin filaments of the animal blastomeres had begun to reorganize into a

**Fig. 4.** Cytokeratin organization in 2-, 4- and 8-cell embryos. By the time of first cleavage (A) the cytokeratin filament system of the vegetal hemisphere has begun to coalesce into a system of anastomosing filament bundles; this reorganization of cytokeratin filaments continues through second (B) and third (D) cleavage to produce a highly regular cytokeratin filament network. The cytokeratin system does not appear to descend into the cleavage furrows (marked by large white arrows in part D). In a negative image of a sectioned, 1h5-labelled, 4-cell embryo (C) (1h5 labelling is black), it is clear that the cytokeratin system is entirely cortical. Bar in part B marks 10μm for all parts.
more extended system, similar to that found in cultured cells and within the vegetal blastomeres (Fig. 5D,E). The cytokeratin filament systems of animal hemisphere cells still differed from those of vegetal hemisphere cells in the size of the cytokeratin meshwork. What appear to be desmosome-type adherence junctions were first observed in both hemispheres of the embryo around this time (see Fig. 5E).

Results of prick activation

In order to study how the pattern of cytokeratin reappearance and reorganization after fertilization is controlled, we examined the effects of prick activation. Prick activation produces many of the same changes in egg structure that are found to follow fertilization. Mature eggs were dejellied and activated by pricking in the animal hemisphere with a clean glass needle. The efficacy of this activation was monitored by watching for the contraction of the animal pigment cap, which was complete by about 5 min after activation (Stewart-Savage & Grey, 1982). Activated eggs were examined at 30, 60, 120 and 180 min after activation. By 30 min postactivation, a punctate pattern of 1h5 labelling was apparent in the vegetal region (Fig. 6A) and occasionally in the animal region (not shown). By 60 min postactivation (Fig. 6B,C), punctate 1h5 labelling was observed in the animal region; both dense aggregates and aggregates composed of short filaments were found. In the vegetal hemisphere, the cytokeratin system was well into the process of transformation from punctate to fibrous organization and a gradient of cytokeratin organization was readily apparent (Fig. 6B,C). By 120 min postactivation (Fig. 6D,E), the transformation of the vegetal cytokeratin system into a fibrous network of bundled cytokeratin filaments was complete. The cytokeratin network was very similar in
appearance to that observed in fertilized eggs (compare with Fig. 4). Unlike fertilized eggs, the cytokeratin system of prick-activated eggs did not appear to be stable and by 180 min postactivation it had begun to break up into dense aggregates (Fig. 6F).

**Discussion**

The cortex of the amphibian oocytes and egg, as apparent from the distribution of pigment granules, is clearly an asymmetric structure. However, aside from the clear asymmetry in pigment distribution, little else is known about cortical asymmetry; likewise, the manner in which cortical asymmetry is generated and maintained remains obscure. One of the major components of the cortex of *Xenopus* oocytes/eggs is cytokeratin-type intermediate filaments (Franz et al. 1983; Gall et al. 1983; Godsave et al. 1984). Intermediate filaments are characteristically insoluble, 8–10 nm filaments that are found in most of the cells of higher eukaryotic organisms (for review see Traub, 1985).

Exploiting the observation of Franz et al. (1983) that the *Xenopus* kidney epithelia-derived cell line A6 contains the same three cytokeratin proteins as the mature *Xenopus* oocyte, we used A6-derived cell residues to generate the monoclonal anti-cytokeratin antibody 1h5; 1h5 reacted specifically with the 56K neutral/basic cytokeratin (Fig. 1). Using 1h5 together with a whole-mount method of indirect immunofluorescence labelling, we have made three unanticipated discoveries concerning the temporal and spatial organization of the cortical cytokeratin system of mature oocytes and early embryos.

The first observation was the high degree of organization characteristic of the vegetal domain of both mature oocytes and early embryos. In the vegetal hemisphere of mature oocytes, the cytokeratin system assumed a highly regular and almost geodesic-type organization. To our knowledge, this type of organization has never before been reported for intermediate filaments of any type, although it bears some resemblance to the organization of actin and

Fig. 5. Animal–vegetal polarity in cytokeratin organization in early embryos. By the 4- to 8-cell stage, the cytokeratin system of the animal region (A) consists of scattered filament fragments; the vegetal cytokeratin network (B) (shown at the same magnification) is dramatically different. In some batches of embryos, the gradient in cortical cytokeratin organization (C) (negative image) from the animal (a) to the vegetal (v) pole included a region in which the network-type organization of cytokeratin filaments appears to break down into cytokeratin aggregates. By stage 6/7, the cytokeratin filaments of the animal hemisphere blastomeres (D) have begun to form integrated filament systems similar to those found in the vegetal blastomeres (E). Arrow in part E marks what appears to be a desmosomal junction. Bar in part D marks 10 μm for parts A,B,D,E; bar in part C marks 10 μm.
actin-associated proteins in spreading fibroblasts (see Lazarides, 1976). How the geometric organization of the cytokeratin system of mature oocytes is generated remains unclear. The cytokeratin system of the vegetal region of early embryos also possessed a highly stereotyped organization, distinctly different from

Fig. 6. Appearance of cytokeratin polarity in prick-activated eggs. Unfertilized eggs were activated by pricking with a clean glass needle. Cytokeratin labelling appeared first as a pattern of spots (A, 30 min postactivation) which later was transformed in the vegetal hemisphere into an anastomosing filament bundle system (B,C, 60 min postactivation). The animal–vegetal gradient in cytokeratin organization is clearly visible in parts B and C. By 120 min postactivation (D,E), the cytokeratin system appeared to be mature and similar to that seen in fertilized eggs. In part D, the cortex has ripped and folded over itself, revealing that the bulk of the cytokeratin system is located in the cortex (white arrows mark limit of folded over region). By 180 min after activation (F), the cytokeratin system appeared to be breaking down into a more amorphous type of organization. Bar in part A marks 100 μm for parts A,C,E,F; bar in part D marks 100 μm for parts B,D.
the 'geodesic'-type cytokeratin organization found in mature oocytes. In early embryos, cytokeratin filaments form large anastomosing bundles; this fishnet-like cytokeratin system covers the entire vegetal region of the embryo. Except for its dimensions, this type of cytokeratin organization appears similar to that characteristically observed in cultured epithelial cells (compare Figs 1, 4, 5D,E).

Our second observation was the polarity in the organization of cortical cytokeratin filaments in both the mature oocyte (Fig. 2) and the early embryo (Fig. 5). In both, the cytokeratin system of the animal region appeared to be either disorganized or organized along completely different lines than the cytokeratin system of the vegetal region. Particularly in the early embryo, it is possible that the cytokeratin filaments present in the animal region are organized into a network of fine filaments and that this network is obscured by the high density of pigment granules present. Nevertheless, it is clear that there exists a gradient in cytokeratin filament organization from the animal to the vegetal pole in early embryos (Fig. 5C).

The third observation concerns the temporal changes in the organization of the cortical cytokeratin system. The transition between oocyte and embryonic-type cytokeratin organization begins during the process of egg maturation (see Fig. 7). A number of profound changes occur within the egg during this period, including the breakdown of the germinal vesicle and the release of its contents into the cytoplasm (see Brachet et al. 1970; Gerhart, 1980). With egg maturation, all traces of the geometric cytokeratin system of the oocyte disappear; only isolated cytokeratin filament fragments and amorphous masses of 1h5-labelled, presumably reorganized cytokeratin-containing material were visible (Fig. 3A,B). Changes in cytokeratin organization with oocyte maturation have been noted previously by Godsave et al. (1984).

With fertilization, the cytokeratin system of the egg undergoes a second structural transformation; cytokeratin organization first becomes punctate and then filamentous (Fig. 3), forming large filament bundles in the vegetal hemisphere by the time of first to second cleavage (Fig. 4). The same spatial and temporal pattern of changing cytokeratin organization was observed when eggs were activated by pricking with a clean glass needle (Fig. 6). This result indicates that the reappearance of 1h5-labelling and the reorganization of cytokeratin filaments is triggered in some manner by the event of activation. Elison (1983, 1985) has reported a number of concerted changes in the mechanical properties of both endoplasm and cortex, as well as in the organization of tubulin through the period leading up to first cleavage. Whether there is any significant correlation between the changes in cytoplasmic consistency and cytokeratin organization remains unclear.

Changes in cytokeratin organization have been observed during mitosis in some cultured epithelial cell lines (Franke, Schmid, Grund & Geiger, 1982; Franke et al. 1983; Lane, Goodman & Trejdosiewicz, 1982) although the molecular mechanism(s) underlying these changes remains obscure. The effects of intracellularly injected monoclonal anti-cytokeratin antibodies (Klymkowsky, 1982; Lane & Klymkowsky, 1982; Klymkowsky et al. 1983) suggest that the binding of antibodies, and presumably other proteins as well, to cytokeratin filaments can dramatically affect cytokeratin organization. It is possible...
that nuclear proteins released at germinal vesicle breakdown (Dreyer, Singer & Hausen, 1981; Hausen, Wang, Dreyer & Stick, 1985) bind to and affect the organization of the egg cytokeratin system. An alternative, and not necessarily incompatible, hypothesis is that changes in cytokeratin organization may be due to posttranslational modification of the cytokeratin proteins themselves; such modification could affect either the organization of cytokeratin filaments or the binding efficiency of 1h5 to the 56K cytokeratin protein, or both. In their analysis of oocytes and laid eggs, Franz et al. (1983) observed changes in the relative amounts of the three cytokeratin proteins present in cytoskeletal preparation of eggs compared with oocytes (see their fig. 5); whether this reflects significant changes in the solubility/organization of the egg cytokeratin system remains to be determined. In any case, our results leave no doubt that, through the period of oocyte/egg maturation and activation/fertilization, the cytokeratin system of the oocyte/egg undergoes a complex series of changes, whose end result is the transformation of the geodesic-type organization of cytokeratin filaments of the oocyte into the polar, fishnet-type cytokeratin filament organization of the embryo.

The similar appearance of asymmetric cytokeratin organization in sperm- and prick-activated eggs provides strong evidence that the cortical asymmetry is maternally preprogrammed. This asymmetry in cortical cytokeratin organization presumably reflects the polar asymmetry of the cortical cytoskeleton as a whole. Such cortical cytoskeletal asymmetry may simply be a manifestation of the molecular asymmetry of the egg (see Carpenter & King, 1982; Hausen et al. 1985; Wylie et al. 1985; Smith, Neff & Malacinski, 1986; Rebagnoli, Weeks, Harvey & Melton, 1985). Alternatively, it may play a number of functional roles in the process of early development, ranging from the determination and/or maintenance of the molecular asymmetry to differential effects on the mechanical properties of the embryonic surface (see Gerhart & Keller, 1986). It is our hope that the intraembryonic injection of the 1h5 antibody will resolve this question unambiguously.

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References


Cytoskeletal asymmetry in the Xenopus embryo


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