Localized Maternal Proteins in *Xenopus* Revealed by Subtractive Immunization

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It has long been appreciated that the localization of cytoplasmic determinants in the egg can provide the foundation for patterning in the embryo. Differences in cell fate among the early blastomeres are thus a consequence of asymmetric distributions of informational molecules prior to fertilization. The frog egg has a single axis of asymmetry present prior to fertilization, the animal/vegetal axis, and the localization of developmental information appears to be polarized along this axis. Such developmental information can be localized as either RNA or protein; localized RNAs are well documented in the *Xenopus* oocyte, and some are thought to play roles in axial patterning. While it is apparent that not all of the localized maternal components are RNAs, much less is known about maternal proteins that might be localized in the egg. In the present study, we have taken a novel approach to identify localized maternal proteins within the *Xenopus* egg. Using a subtractive immunization strategy, we have generated monoclonal antibodies which recognize antigens that are restricted to the vegetal cortex of fertilized eggs. Analysis of biogenesis during oogenesis reveals two distinct patterns of localization to the cortex. At least three of these localized antigens are proteins, and these localized proteins could represent maternal determinants with roles in patterning.   © 1997 Academic Press

INTRODUCTION

The detailed studies of cell lineage by early cytologists such as C. O. Whitman, E. B Wilson, and E. G. Conklin (Whitman, 1878; Conklin, 1905; reviewed in Wilson, 1925), have led to the proposal that the localization of maternal factors in the egg provides the basis for pattern formation in the embryo. According to this view, maternal components become regionally localized in the oocyte during oogenesis and are subsequently inherited differentially during cleavage.

The localization of maternal factors in the frog egg can be inferred from an analysis of the developmental potential along the animal/vegetal axis (AV). The egg has a single axis of asymmetry present prior to fertilization, the AV axis, along which the localization of macromolecules appears to be polarized (reviewed in Klymkowsky and Karnovsky, 1994). The developmental potential along the AV axis is recapitulated in the fate map of the three primary germ layers, the ectoderm, the mesoderm, and the endoderm. The animal hemisphere gives rise to ectodermal cell types, while the vegetal hemisphere contains prospective endoderm, and mesoderm results from an inductive signal emanating from the vegetal blastomeres received by the overlying cells in the marginal zone (Nieuwkoop, 1969). Also localized to the vegetal hemisphere is the germ plasm, which is an aggregation of mitochondria and granular mate-

rial believed to contain protein and RNA (Williams and Smith, 1971). Early in oogenesis, germ plasm accumulates in the mitochondrial cloud, or Balbiani body, and moves to the vegetal cortex after Balbiani body breakdown during stage II of oogenesis (Heasman *et al.,* 1984). In the egg, germ plasm is restricted to the vegetal cortex and is inherited preferentially by the primordial germ cells during cleavage, suggesting that it acts as a localized maternal germline determinant (Blackler, 1958).

Maternal determinants can be localized as either RNA or protein. In the frog egg, mRNAs have been identified that localize to either the animal pole or the vegetal pole, and certain of these mRNAs have been proposed to be critical for patterning of the embryo (reviewed in St. Johnston, 1995). Much less is known, however, about maternal proteins that might be localized in the frog egg. An early study (Moen and Namenwirth, 1977), looking at AV differences in nonyolk proteins by gel electrophoresis, was limited by the fractionation methods, in that only 4% of the egg's total nonyolk proteins could be loaded on a gel. Even within this small subset of maternal proteins, a significant number appeared to be differentially distributed along the AV axis. Independent attempts have been made to raise monoclonal antibodies (MAbs) to vegetal hemisphere nonyolk proteins (Smith *et al.,* 1986) and animal hemisphere nonyolk cytoplasm (Suzuki *et al.,* 1991). Surprisingly, both approaches

identified MAbs which react with animal hemisphere antigens. A possible explanation for this is that the fractionation methods used to remove yolk proteins may have also removed proteins bound to the vegetal cortex and/or cytoskeleton (Elinson *et al.,* 1993).

In the present study, we have taken an novel immunological approach to identify localized maternal proteins within the frog egg. A subtractive library of MAbs against proteins localized to the vegetal hemisphere was constructed. The vegetal hemisphere was targeted due to the abundance of developmental information residing there. Subtractive immunization was used to remove the representation of animal hemisphere antigens in the hybridoma population. Characterization by immunocytochemistry and Western blotting indicates that we have identified at least three proteins which are localized to the vegetal hemisphere. These localized proteins may represent maternal determinants with roles in patterning and polarity.

MATERIALS AND METHODS

Oocytes, Eggs, and Embryos

Oocytes were obtained surgically from adult female *Xenopus laevis* frogs (Xenopus I) and defolliculated by treatment with 0.2% collagenase (Sigma, Type I) in OR2− (82 mM NaCl, 2.5 mM KCl, 1.5 mM Na$_2$HPO$_4$, 50 mM Hepes, pH 7.6). Fertilized eggs were obtained from adult females that were induced to spawn with 800 units of human chorionic gonadatropin (HCG, Sigma), delivered to the dorsal lymph sac. In some cases, females were primed with 30 units of pregnant mare serum gonadatropin (Sigma), 3 to 4 days in advance of HCG treatment. Eggs were manually squeezed from spawning females into a dry petri dish. Testes were dissected from a male and held in MMR (100 mM NaCl, 2 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM Hepes, pH 7.4) plus 0.2 mg/ml gentamicin at 4°C, until needed. For fertilization, one-quarter of a testis was macerated in MMR in a microcentrifuge tube and held on ice. A few drops of testis suspension were placed on spawned eggs, and the petri dish was flooded with one-third strength MMR (MMR/3). Approximately 10 min after fertilization, eggs were dejellied in 2.5% cysteine in MMR/3 (pH 8.0), with gentle shaking. Cysteine was removed with three washes in MMR/3. Fertilizations and development of embryos occurred at 18 to 21°C. Oocytes were staged as in Dumont (1971), and embryos were staged according to Nieuwkoop and Faber (1967).

Immunosubtraction and Immunization

Antigen was prepared by sectioning frozen eggs into animal and vegetal thirds. Individual fertilized eggs, taken throughout the second half of the first cell cycle, were oriented on glass slides with the AV axis perpendicular, and excess buffer was removed. The slide was placed on a crushed dry ice surface under a dissecting microscope. As the egg froze, the vegetal third or animal third of the egg was cut off with a scalpel. Because only one-third of the egg was removed, the animal/vegetal fractions were derived from sister eggs, not the same egg. Egg fractions were held at −80°C until use, then thawed at room temperature and sheared by pipetting and vortexing to aid antigen presentation. Samples were diluted in 2 vol of protease inhibitor buffer [0.5 mM Pefabloc (Boehringer-

Mannheim), 20 µg/ml aprotinin (Sigma), 20 µg/ml leupeptin (Sigma), 1 mM EDTA, in phosphate-buffered saline (PBS)], and the protein concentration was determined by the Bradford assay (Bio-Rad). Antigens were suspended in RIBI adjuvant (Immunochemical Research) to a final protein concentration of ~500–700 µg/ml, just prior to immunization.

Balb/cJ mice (Jackson Laboratories) were injected (i.p.) with 200 µg of animal hemisphere extract, followed immediately by cyclophosphamide (Cytoxan, Mead Johnson, 100 mg/kg body wt). Cyclophosphamide treatment was repeated 24 and 48 h postimmunization. Cyclophosphamide was cleared for 2 weeks following the final application, and mice were immunized with vegetal hemisphere extract (300 µg protein in RIBI, i.p.). A booster immunization followed 2 weeks later (300 µg protein in RIBI, i.p.), with a final boost the following 2 weeks (150 µg in 0.15 M NaCl, given via tail vein). Spleens from selected mice were harvested 3 days after the final boost.

The first fusion (D4) and hybridoma selection was performed as in Hixson and Allison (1985), using the parental myeloma cell line PAI. The second fusion (D5) and hybridoma selection was performed using the cell line X63Ag8.653 and the ClonaCell-HY hybridoma cloning kit (Stem Cell Technologies). Selection and cloning were performed on semisolid medium (Davis *et al.,* 1982).

Whole-Mount Immunocytochemistry

Oocytes and eggs were fixed in methanol at room temperature or at −20°C overnight. Eggs were fixed between 0.66 and 0.75 of the first cell cycle. Samples were rehydrated in a methanol series and washed three times for 20 min each in PBS with 0.1% Tween 20 (PBS/T20). Samples were incubated overnight at 4°C in primary antibody plus an equal volume of PBS/T20 with 2% fetal calf serum, followed by two washes of PBS/T20 over 1 h. Secondary antibody [fluoresceinated anti-mouse IgG (Sigma) or Cy3-labeled anti-mouse IgG (Jackson ImmunoResearch)] was applied diluted in PBS/T20 plus 2% fetal calf serum. Secondary antibodies were washed with two changes of PBS/T20 at room temperature. For vital staining of germ plasm, fertilized eggs were bathed in the mitochondrion-selective dye Red CMXRos (MitoTracker, Molecular Probes), 1–2 nM in MMR/3 for 1 h followed by several washes in MMR/3, as in Robb *et al.* (1996), and samples were fixed in methanol. When required, samples were cleared for optical sectioning by two washes of 100% ethanol followed by Murray's clear (1:2, benzyl alcohol:benzyl benzoate). Whole-mounts were analyzed on a Zeiss IM inverted microscope or a Zeiss LSM 410 inverted confocal microscope. Eggs were oriented with the aid of a dissecting microscope mounted over the stage. For analysis of regional localization along the AV axis, eggs were optically sectioned through the animal and vegetal hemispheres, to a depth of 200–300 µm past the surface.

Western Blotting

Fertilized eggs, stored in ethanol, were resuspended directly in sample buffer (5% β-mercaptoethanol; 4% SDS; 5% glycerol; 50 mM Tris, pH 6.8) and boiled. Total proteins (100 µg) were separated by SDS–PAGE and transferred to nitrocellulose as in Towbin *et al.* (1979). Filters were washed in blotto [50 mM Tris (pH 7.5), 185 mM NaCl, 0.05% Tween 20, 3% (w/v) nonfat dry milk] and probed (2 h at room temperature) with MAbs (diluted 1:1 in blotto). After washing for 1 h in blotto, peroxidase-conjugated sheep α-mouse (Cappel) was used as secondary antibody, and detection was by enhanced chemiluminescence (Genius 7, Boehringer).
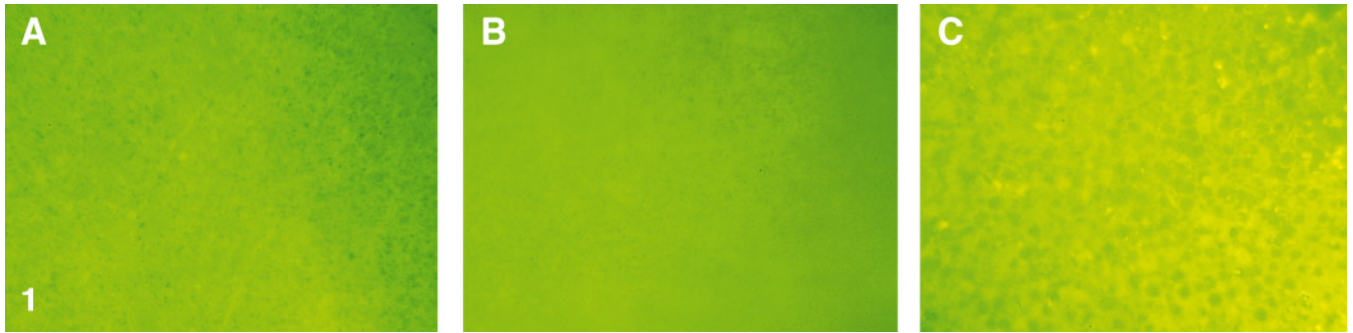
FIG. 1. Immunosuppression reduces the immune response to nonlocalized antigens. Shown are vegetal cortical views of whole-mount preparations of fertilized eggs, stained with (A) preimmune serum; (B) serum from a mouse that was immunized with animal hemisphere extract, and immunosuppressed; and (C) serum from a mouse that was immunized with animal hemisphere extract, no immunosuppression. The photographs were taken on a Zeiss IM inverted microscope using a 40× objective.

RESULTS

A general problem in generating MAbs to regionalized antigens is that they may be missed in the background of nonlocalized antigens or underrepresented in the presence of immunodominant proteins such as yolk. For targeting proteins localized to the vegetal hemisphere, this difficulty was overcome by using a subtractive immunization strategy to selectively eliminate antibody production against antigens that are shared between the animal and vegetal hemisphere. In this strategy, mice are challenged with animal hemisphere cytoplasm, then immunosuppressed with the drug cyclophosphamide. Cyclophosphamide blocks the clonal proliferation of lymphocytes in response to antigen presentation. After clearing of the immunosuppressant, the same animal is then challenged with vegetal hemisphere cytoplasm. The animal is capable of responding to only the unique antigens of the vegetal hemisphere, and the resulting hybridomas are in effect a subtractive library of MAbs, specific to vegetal hemisphere antigens. The technique can result in a significant increase in selection of MAbs against specific antigens (Matthew and Sandrock, 1987; Suzue *et al.,* 1990; Carnahan and Patterson, 1991). However, this approach had not been used for the identification of a large class of localized antigens, and it remains to be determined if immunosubtraction would be effective in a complex, yolk-rich cell such as the frog egg.

Immunosuppression Is Effective in Eliminating Response to Nonlocalized Antigens

To assess immunosuppressed mice for successful subtraction, mice were immunized with animal hemisphere cytoplasm, and a subset were immunosuppressed by treatment with cyclophosphamide. Sera from the mice were then tested against frog eggs by whole-mount immunocytochemistry. Shown in Fig. 1 is a direct comparison of sera from suppressed versus unsuppressed mice. Vegetal views of eggs are presented to ascertain whether immunosuppression could block the response to antigens shared between the animal and vegetal hemispheres (nonlocalized antigens). Preimmune serum (Fig. 1A) exhibits very little staining. The serum shown in Fig. 1B was from a mouse that had been challenged with animal hemisphere extract, then immunosuppressed, and demonstrates staining equivalent to that observed with preimmune serum (Fig. 1A). In contrast, high general staining is evident in Fig. 1C, which shows staining with serum from a mouse that was challenged with animal hemisphere extract, but not subjected to immunosuppression. Sera from 6 of 10 mice, in three separate trials, gave no humoral response to antigen presentation after immunosuppression. Sera from the other 4 mice showed only a weak background staining. The immunosuppressed lymphocyte population can also recover with time. Those immunosuppressed mice not used for further immunizations were maintained for 6 months, and their sera were retested against frog egg whole-mounts. All sera tested exhibited positive staining of the vegetal cortex (data not shown).

These results indicate that immunosuppression is successful in eliminating the response to antigens common to both animal and vegetal hemispheres of the *Xenopus* egg. Only those mice demonstrating successful subtraction (as with Fig. 1B) were chosen for subsequent immunization with vegetal hemisphere extract.

Region-Specific Hybridomas Can Be Generated after Immunosuppression

Two subtraction schemes were completed, followed by two hybridoma fusions. Initial screening of hybridomas was limited to examination of the vegetal hemisphere in whole-mount preparations. For the screening, 20 eggs pooled from three females were scored for each colony. Hybridomas exhibiting positive staining of the vegetal cortex were stored frozen until the initial screen was complete and further analysis could be performed. For the first fusion, D4, primary screening revealed positive staining in 23 of the 288 primary colonies. For the second fusion, D5, colonies were
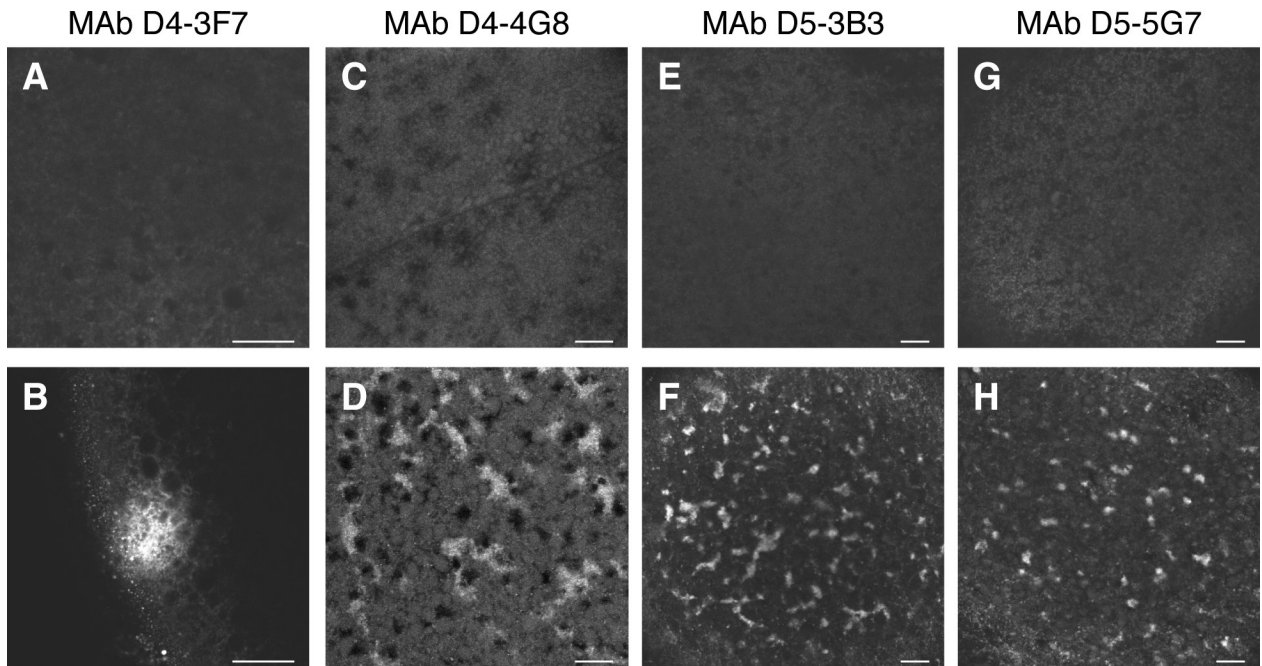
MAb D4-3F7   MAb D4-4G8   MAb D5-3B3   MAb D5-5G7

FIG. 2. Vegetally restricted antigens are revealed by staining of fertilized eggs with MAbs. (A) D4-3F7, animal view. (B) D4-3F7, vegetal view. (C) D4-4G8, animal view. (D) D4-4G8, vegetal view. (E) D5-3B3, animal view. (F) D5-3B3, vegetal view. (G) D5-5G7, animal view. (H) D5-5G7, vegetal view. All eggs were albinos, cleared for optical sectioning, and the views are ~15 μm from the plasma membrane. The scale bars represent 20 μm, and the images were obtained using a Zeiss confocal microscope.

screened in pairs and positive vegetal staining was found for 53 pairs of colonies of a total of 527. For the two separate fusions, a minimum of 9% of the hybridomas generated were positive. These results demonstrate that after clearing of the immunosuppressant, treated mice are able to mount an immune response.

To ascertain regional specificity along the AV axis, staining in both the vegetal and animal hemispheres of albino eggs was examined with seven hybridomas, which were chosen based on their interesting staining patterns in the initial screen. Three hybridomas were eliminated as not producing antibodies specific to epitopes in the vegetal cortex. The four remaining hybridoma lines produce antibodies which recognize antigens that are restricted to the vegetal hemisphere, and their characterization is presented below in further detail.

Hybridomas D4-3F7, D4-4G8, D5-3B3, and D5-5G7 Recognize Antigens That Are Localized to the Vegetal Cortex

Staining of fertilized eggs with medium from hybridoma D4-3F7 reveals large patches that are restricted to the vegetal cortex. The patches are ~20–40 μm across and number between 2 and 10 per egg. A single such patch is shown in Fig. 2B. Each patch appears as a somewhat diffuse particulate region, intercalating between yolk platelets; the antigenic material does not appear to displace yolk platelets

or reside in a yolk-free region, nor are the yolk platelets themselves stained. No staining is detectable in the animal hemisphere, as demonstrated in Fig. 2A, which shows an animal hemisphere view of the same egg as in Fig 2B.

Fertilized eggs stained with MAbs from hybridoma D4-4G8 show distinct reticulated areas confined to the vegetal cortex. This is illustrated in Fig. 2D, where staining is evident in a view of the vegetal cortex of a fertilized egg, while a view of the animal hemisphere of the same egg shows no staining (Fig. 2C). Based on optical sectioning of cleared eggs, the staining is confined to the vegetal cortical and subcortical region, up to 50 μm past the plasma membrane.

Hybridoma D5-3B3 produces MAbs that recognize an antigen which is present in the vegetal cortex (Fig. 2F) and absent from the animal cortex, as demonstrated in Fig. 2E, which shows staining of the same fertilized egg shown in Fig. 2F. The D5-3B3 epitope is distributed as numerous patches, which are also reticular in appearance and are confined to the cortical and subcortical region of the vegetal hemisphere, to a similar depth as for D4-4G8.

The epitope revealed by staining with MAbs from D5-5G7 is also restricted to the cortex and subcortex of the vegetal hemisphere (Fig. 2H), with staining absent from the animal hemisphere (Fig. 2G). In size, shape, and distribution, the D5-5G7 epitope is similar to both the D4-4G8 and D5-3B3 epitopes, in that it exists as reticulated areas in the vegetal cortex, extending past the plasma membrane to approximately 50 μm.

MAbs D5-3B3, D4-4G8, and D5-5G7 Recognize Antigens Which Colocalize with Germ Plasm

The patterns of staining revealed by MAbs D4-4G8, D5-3B3, and D5-5G7 in fertilized eggs are reminiscent of recently published images of germ plasm in the frog egg (Robb *et al.,* 1996). To examine the possibility that the vegetal-specific MAbs recognize a component localized to the same subcellular compartment as the germ plasm, fertilized eggs were labeled with the vital dye, MitoTracker, which stains the germ plasm (Robb *et al.,* 1996). The labeled eggs were then fixed and costained with MAbs. Staining with each of the three MAbs was seen to colocalize with the germ plasm, as shown in Fig. 3, where the green images (Figs. 3A, 3D, and 3G) show staining with the MAbs, the red images show MitoTracker labeling (Figs. 3B, 3E, and 3H), and the overlap is shown by yellow (Figs. 3C, 3F, and 3I). In the egg, colocalization with islands of germ plasm is striking, but not perfectly coherent for D5-3B3 (Fig. 3C), D4-4G8 (Fig. 3F), and D5-5G7 (not shown). Regions of overlap (yellow) predominate, but small regions of contiguous nonoverlapping staining (green or red) are present as well. This pattern may indicate a complex of two overlapping compartments. During the first cell cycle, islands of germ plasm coalesce into larger islands, which eventually ingress inward along early cleavage furrows (Ressom and Dixon, 1988; Savage and Danilchik, 1993). All three epitopes display such behavior, which is demonstrated with MAb D5-5G7 (Figs. 3G–3I), in an eight-cell embryo showing a large coalesced island of germ plasm adjacent to a cleavage furrow, costained with D5-5G7 (Fig. 3G) and MitoTracker (Fig. 3H). Colocalization, as demonstrated by an image showing both channels (Fig. 3I), is very coherent at this stage and suggests that there may be remodeling of the D5-5G7, D4-4G8, and D5-3B3 epitopes during aggregation.

Two Patterns of Biogenesis Are Revealed by MAbs D5-3B3, D5-5G7, and D4-4G8

The colocalization of D4-3B3, D4-4G8, and D5-5G7 antigens with germ plasm in eggs led us to ask if the biogenesis of these antigens is similar to the pattern reported for germ plasm during oogenesis. In very young (stage I) oocytes, germ plasm is localized to the Balbiani body (Heasman *et al.,* 1984). The epitopes recognized by MAbs D5-3B3 and D5-5G7 are both present as early as stage I of oogenesis and are highly localized to the Balbiani body. This is demonstrated in a stage I oocyte stained with D5-5G7 in Figs. 4A and 4B, showing serial sections through the Balbiani body. MAb D5-3B3 exhibits the same pattern, as seen in Fig. 4C. During stage II of oogenesis, the Balbiani body breaks down and its contents migrate to the future vegetal cortex (Heasman *et al.,* 1984). The epitopes for MAbs D5-3B3 and D5-5G7 follow this pattern, as demonstrated for D5-3B3 in Fig. 4D. In this stage II oocyte the Balbiani body is no longer present, and the staining material now extends between the GV and the vegetal cortex. MAb D5-5G7 demonstrates the same pattern (data not shown). In contrast to the Balbiani

body localization seen for these antigens, no epitope could be detected by immunocytochemistry with MAb D4-4G8 until midoogenesis. An example of the lack of staining in a stage I oocyte is shown in Fig. 4E, followed by an example of cortical staining in a stage III oocyte in Fig. 4F, the earliest stage at which the epitope is detectable by immunocytochemistry.

MAbs D4-4G8, D4-3F7, and D5-5G7 Recognize Different Protein Antigens

To determine whether the vegetal-specific MAbs recognize protein antigens, Western blot analysis was performed against total fertilized egg extracts. As shown in Fig. 5, MAbs D4-3F7, D4-4G8, and D5-5G7 each recognize a single protein antigen of ~50 kDa for D4-4G8 (lane 1), ~40 kDa for D4-3F7 (lane 2), and ~67 kDa for D5-5G7 (lane 3). The D5-3B3 antigen is undetectable on Western blots (not shown), suggesting either that D5-3B3 does not recognize a protein epitope or that it cannot recognize the antigen after denaturation. The Western blotting results with MAbs D4-3F7, D4-4G8, and D5-5G7 demonstrate that these three different hybridoma lines produce antibodies against different vegetally localized proteins.

DISCUSSION

Reasoning that localized maternal molecules play a significant role in development, we created a subtractive library of MAbs against proteins specific to the vegetal hemisphere of the frog egg. Most current analyses of maternally derived patterning in the frog embryo have been the result of a top-down strategy, wherein determined cell types are the starting point for a reverse mapping of the signaling pathways involved (reviewed in Kessler and Melton, 1994; Hogan, 1996; Kuhl and Wedlich, 1997). This approach has significantly increased our understanding of early patterning in the embryo, but mapping has led back to the egg in only a few cases (see for examples, Ku and Melton, 1993; Heasman *et al.,* 1994; Yost *et al.,* 1995; Larabell *et al.,* 1997). Complementary approaches to directly identify determinants within the egg or oocyte have also been successful (Rebagliati *et al.,* 1985; Mosquera *et al.,* 1993), but have focused attention exclusively on localized RNAs rather than proteins. To search for localized maternal proteins with potential roles as determinants, we have used the egg as a starting point for a novel immunological approach to this problem. We have discovered at least three proteins which are polarized along the AV axis, all three being highly localized to the vegetal cortex.

The means to uncover potentially rare, localized antigens against a background of abundant unlocalized proteins has been provided by an immunosubtraction strategy (Matthew and Sandrock, 1987). It was not immediately obvious, however, that such a scheme could be successfully applied to a large, yolk-rich cell such as the frog egg, which has extensive stockpiles of maternal proteins. Therefore, it was im-
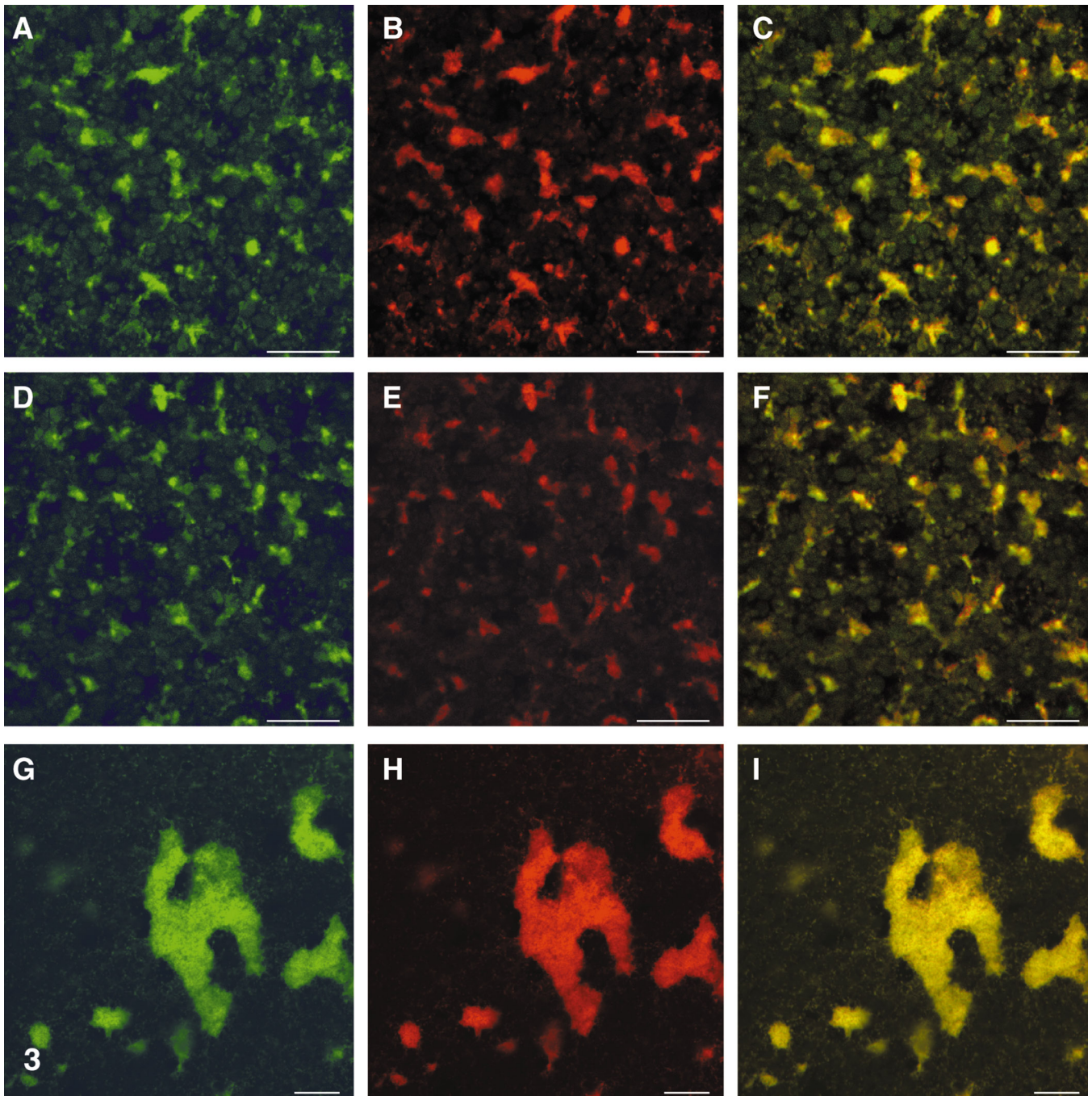
FIG. 3. Colocalization of vegetal antigens with germ plasm. Shown are vegetal views of fertilized eggs (A–F) or an eight-cell embryo (G–I) labeled with MitoTracker and stained with the following MAbs: (A–C) D5-3B3, (B–F) D4-4G8, and (G–I) D5-5G7. MAb staining is shown as green, germ plasm is shown in red, and regions of colocalization appear as yellow. All eggs and embryos were cleared for optical sectioning, and the images were obtained using a Zeiss confocal microscope. The scale bars represent 20 μm.

portant to derive a minimum estimate of the effectiveness for the immunosubtraction. Our results indicate that subtraction was effective, but not complete, since three of the seven MAbs examined in detail were not specific to the vegetal hemisphere. Also, in the initial screen 8 of the total 815 hybridomas could be eliminated as not vegetally specific, since staining was strong enough to be seen even in the pigmented animal hemisphere. These data indicate that
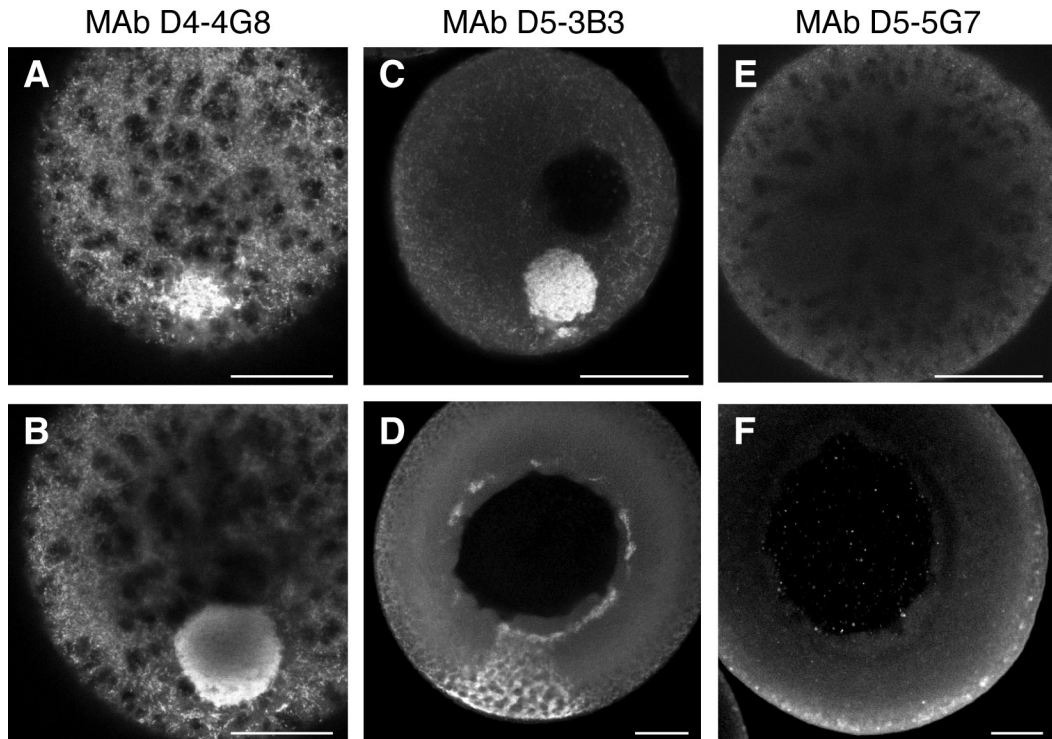
MAb D4-4G8   MAb D5-3B3   MAb D5-5G7

FIG. 4. Two distinct patterns of biogenesis during early oogenesis. Oocytes were stained with the indicated MAbs and imaged using a Zeiss confocal microscope. Scale bars for all panels represent 50 μm. (A, B) Serial sections of a stage I oocyte, stained with D5-5G7. (C) Stage I oocyte stained with D5-3B3. (D) Stage II oocyte stained with D5-3B3. (E) Stage I oocyte stained with D4-4G8. (F) Stage III oocyte stained with D4-4G8.

a minimum of 0.5% (4/815) of the hybridomas generated through immunosubtraction are genuine products of subtraction. Since spleen cell/myeloma fusions typically yield
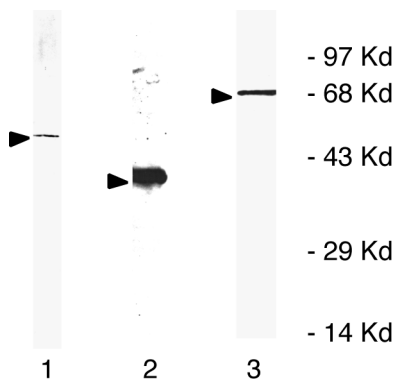

FIG. 5. Protein antigens are revealed by Western blot analysis. Egg extracts were subjected to SDS–PAGE, transferred to nitrocellulose filters, and probed with D4-4G8 (lane 1), D4-3F7, (lane 2), or D5-5G7 (lane 3). The positions of the proteins recognized by the MAbs are indicated by arrowheads, and the sizes of molecular weight standards are shown at the right.

between 300 and 600 colonies, a minimum result of 0.5% subtracted positives will yield at least two to three MAbs specific to the region of interest, a success rate which supports the use of this strategy to identify specific localized proteins.

We have identified four hybridomas whose MAbs recognize epitopes showing strong localization to the vegetal cortex of the frog egg. One MAb, D4-3F7, recognizes a protein with a previously uncharacterized distribution in the vegetal cortex. The other three MAbs recognize epitopes which colocalize to islands of germ plasm, a long-studied cytoplasmic localization thought to be a germ cell determinant. Two of the epitopes which colocalize with the germ plasm, those recognized by D5-3B3 and D5-5G7, are present in the Balbiani body of early oocytes, while epitope D4-4G8 is not identifiable until stage III of oogenesis. Based on Western blot analysis, the MAbs recognize at least three distinct proteins of ~40 (D4-3F7), ~50 (D4-4G8), and ~67 kDa (D5-5G7). In spite of the similarities in staining patterns seen by whole-mount immunocytochemistry with MAbs D4-4G8, D5-3B3, and D5-5G7, it is apparent from Western blots, and the differences in their biogenesis, that MAb D4-4G8 recognizes a different protein than MAb D5-5G7. The staining patterns, however, would suggest that the two proteins are localized to the same compartment in the egg. MAb D5-

3B3 may also recognize a unique antigen which localizes to this compartment, or it may recognize a different epitope of the same protein as recognized by MAb D5-5G7.

The identity of the proteins recognized by the vegetal-specific MAbs remains to be established. There is nothing immediately obvious about the nature of the D4-3F7 epitope as seen by immunohistology. No previously described structures in the vegetal cortex of the frog egg are known to us to be comparable, nor does the size of the protein recognized by Western blot recommend an identity. Further characterization of the biogenesis of the protein, and its patterning in embryogenesis, will be necessary to provide clues to its possible function or identity.

The colocalization of the D4-4G8, D5-3B3, and D5-5G7 epitopes with islands of germ plasm is intriguing. Germ plasm is historically defined as electron-dense, granular–fibrillar material embedded in aggregates of mitochondria, with no limiting membrane (Smith, 1975). This picture is becoming more complex. From the work of Robb *et al.* (1996), it is clear that the compartments of mitochondria stained by MitoTracker encompass other molecules, in particular a kinesin-like protein (Xklp1), vimentin, and microtubules. The presence of these molecules, which are not unique to the germ plasm, indicates that islands of germ plasm may contain more than the classic electron-dense material surrounded by mitochondria. Additional precedence for this is provided by Terasaki *et al.* (1984), who showed that staining of mitochondria with the lipophilic dye $DiOC_6(3)$ also reveals a strong association with the endoplasmic reticulum. In *Caenorhabditis elegans,* transient associations between the germ plasm and specific proteins have been demonstrated. Both MEX-3, a cytoplasmic putative RNA binding protein, and PIE-1, a potential repressor of transcription, associate with the germ-line-specific P granules only during early development (Draper *et al.,* 1996; Mello *et al.,* 1996). MEX-1, which is similar to PIE-1 in structure, associates with P granules only during early cleavages and may play a role in anchoring P granules for proper partitioning (Guedes and Priess, 1997). A similar transient association with germ plasm may explain our results.

We do not believe that MAbs D4-4G8, D5-3B3, and D5-57 recognize germ plasm components in the classic sense, but instead that the staining patterns seen with these MAbs reveal overlapping subcompartments. The first appearance of D4-4G8 staining during stage III of oogenesis strongly argues against its identity as a germ plasm component, since no such pattern has been described for any germ plasm element. While the early biogenesis of the D5-3B3 and D5-5G7 epitopes is similar to that described for germ plasm, costaining with MitoTracker in the fertilized egg is not perfectly coherent, suggesting that they are not germ plasm components. Rather, the staining patterns seen with MAbs D4-4G8, D5-3B3, and D5-57 and MitoTracker reveal two closely associated compartments in the vegetal cortex of the egg. It is only during cleavage, as the germ plasm islands aggregate, that these compartments appear to coalesce into one coherent compartment. Localization to distinct, over-lapping domains within the vegetal cortex has been described for RNA molecules (Forristall *et al.,* 1995; Kloc and Etkin, 1995), and our results indicate that the organizational complexity of the vegetal cortex may be even greater than previously appreciated.

It is evident that many maternal components of the frog egg are localized in a manner critical to the correct patterning of the future embryo. The vegetally localized maternal proteins that we have identified may contribute to this, and it will next be important to determine what role these proteins may play in early embryogenesis. Our results suggest that the analysis of localized maternal proteins in the frog egg is a productive area for exploring potential determinants.

ACKNOWLEDGMENTS

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