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Xenopus Cdc6 confers sperm binding competence to oocytes without inducing their maturation

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ABSTRACT **Amphibian eggs normally require meiotic maturation to be competent for fertilization. A necessary prerequisite for this event is sperm binding, and we show that under normal physiological conditions this property is acquired at, but not before, meiotic maturation. Immature oocytes do not bind sperm, but injection of total egg poly(A)$^+$ mRNA into immature oocytes confers sperm binding in the absence of meiotic maturation. Using an expression cloning approach we have isolated a single cDNA from egg poly(A)$^+$ mRNA that can induce sperm binding in immature oocytes. The cDNA was found to encode *Xenopus* Cdc6, a protein that previously has been shown to function in initiation of DNA replication and cell cycle control. This unanticipated finding provides evidence of a link between a regulator of the cell cycle and alterations in cell surface properties that affect gamete binding.**

Progesterone-induced meiotic maturation is a crucial step in the development of fertilizability in *Xenopus* oocytes. The fully grown, stage 6 oocyte that is arrested in prophase of meiosis I cannot be fertilized by sperm and fails to be activated when pricked with a needle or exposed to calcium ionophore A23187 (1). *In vivo* the follicle cells surrounding the oocyte secrete progesterone, which stimulates the oocyte to resume meiosis and undergo germinal vesicle breakdown (GVBD), chromosome condensation, and assembly of the meiosis I spindle. After a highly asymmetrical cell division that generates the first polar body, the oocyte enters meiosis II and arrests at metaphase (reviewed in refs. 2 and 3). During the process of meiotic maturation, significant increases have been observed in protein synthesis and in posttranslational protein modifications, such as protein phosphorylation and dephosphorylation, which are believed to be necessary for the formation of a fertilizable egg (1, 4–6). As the maturation program unfolds, the cortex of the oocyte, which consists of the oocyte envelope, plasma membrane, and structures immediately underneath the plasma membrane, is greatly altered. Major ultrastructural changes that require new protein synthesis take place (1, 7, 8) and affect the enzymatic, transport, electrical, and contractile properties of the cortex (1, 9–12). As a result, the oocyte acquires the ability to propagate a wave of cortical granule exocytosis in response to the activation signals and the ability to block polyspermy. The capacity of the oocyte to initiate DNA replication also appears during maturation, possibly as a result of accumulation and posttranslational modifications of components required for this process (13).

The meiotic maturation process takes about 5–6 hr after which the mature oocytes are ovulated into the coelomic cavity of the frog. The resulting coelomic eggs can be activated by ionophore or pricking and can be fertilized by sperm to yield normal embryos if the coelomic egg envelope (CE) is removed (14). This removal is required because sperm cannot penetrate the CE (15). The transformation of the CE to the sperm-receptive vitelline envelope (VE) of the oviposited egg occurs when the coelomic egg passes through the oviduct. This process involves ultrastructural changes in the envelope and processing of a major 43-kDa glycoprotein (gp43) in the CE to 41 kDa by a protease secreted from the oviduct (8, 16, 17). After several hours the coelomic egg passes through the oviduct and emerges as an oviposited egg. Subsequent fertilization releases the egg from its metaphase arrest and allows it to complete meiosis II and enter mitosis.

The detailed mechanism of fertilization in *Xenopus* remains to be elucidated (reviewed in refs. 18 and 19). As a first step in identifying at the molecular level the components involved in this process, we have identified a pair of related glycoproteins in the egg vitelline envelope, gp69/64, that play a key role in sperm binding (20). We also have found that although gp69/64 is present in the envelopes of oocytes and coelomic eggs, it is not accessible to polyclonal anti-gp69/64 antibodies or to sperm. Therefore, sperm do not bind to oocytes or coelomic eggs. Instead, sperm only bind to oviposited eggs, in which gp69/64 is exposed on the surface (21). Evidence that a disintegrin on *Xenopus* sperm interacts with the plasma membrane of the egg has been reported very recently (22).

In the present study, we found that without meiotic maturation passage through the oviduct is not sufficient to induce immature oocytes to bind sperm, suggesting that the earlier surface changes that take place during meiotic maturation are a prerequisite to sperm binding. Assuming that some of these changes are caused by new protein synthesis, we sought to identify these factors that conferred sperm binding ability to the oocyte. We found that microinjection of total egg poly(A)$^+$ mRNA obviates the necessity of the progesterone-induced maturation step in inducing sperm binding, providing that the oocytes have passed through the oviduct. Based on this finding, we developed an expression cloning approach and identified a mRNA that can induce sperm binding to oocytes while not inducing their complete maturation.

MATERIALS AND METHODS

Oocyte Manipulations. *Xenopus laevis* was purchased from Nasco, and segments of ovaries were surgically removed from unstimulated females (females not injected with human chorionic gonadotropin for at least 4–6 weeks) under hypothermic anesthesia. Oocytes were isolated and defolliculated by a brief (0.5-hr) treatment of the ovary segments with 0.5% collagenase (type I, Sigma) in OR2 medium (23), followed by manual removal of the follicular layer with forceps. Isolated oocytes were transferred to a fresh Petri dish and washed with fresh OR2 medium on a shaker for 3 × 1 hr to remove the remaining follicle cells. Isolated oocytes were incubated in OR2 medium supplemented with 0.1

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Abbreviations: GVBD, germinal vesicle breakdown; CE, coelomic egg envelope; VE, vitelline envelope; CPE, cytoplasmic polyadenylation element; UTR, untranslated region.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. U76834).

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mg/ml of gentamycin (GIBCO) at 18°C overnight before being used in sperm binding assay. Oocytes after these steps were free of follicle cells. To induce maturation, oocytes were exposed to progesterone (Sigma) at 1 μg/ml for ≈6 hr until GVBD had occurred.

Sperm Binding Assay and *in Vitro* Fertilization Assay. Sperm-oocyte binding assays and *in vitro* fertilization assays were performed as previously described (20).

Polyclonal Antibodies. Rabbit antisera were raised against purified gp69 and gp64. Antibodies were partially purified by ammonium sulfate precipitation and DEAE-matrix chromatography. This antibody was named anti-VE antibody because it reacts, in addition to gp69/64, with gp120/112, gp41, and weakly with gp37. After preadsorption with gp120/112, gp41, and gp37 that had been covalently linked to Affigel-15 (Bio-Rad), the resulting antibody, named anti-gp69/64 antibody, specifically recognizes only gp69 and gp64 glycoproteins in both their denatured form and their native form in the VE (20).

Western Blot and Immunofluorescent Staining. Immunological procedures were performed as previously described (21).

Egg cDNA Library Construction and mRNA Transcription. Total RNA was isolated from unfertilized, oviposited eggs as described by Evans and Kay (24). Poly(A)$^+$ mRNA was purified from total RNA using Dynabeads and reagents from Dynal (Oslo, Norway). An unidirectional egg cDNA library was constructed starting from 5 μg of egg poly(A)$^+$ mRNA using a ZAP express cDNA synthesis and cloning kit (Stratagene). The primary lambda phage library was amplified once, and an aliquot of the library was transformed into a phagmid library by mass *in vivo* excision. Plasmid DNAs purified from pools of clones from the phagmid library were linearized by *Not*I restriction digestion and used as templates for mRNA synthesis using T3 promoter. Capped mRNAs were synthesized by using an Ambion (Austin, TX) *in vitro* transcription kit.

mRNA Microinjection and Expression Cloning. Oocytes were microinjected at the equatorial zone with 10–40 ng of mRNA (40 nl) dissolved in RNase-free ddH_2O and incubated in oocyte culture medium (14) at 18°C for ≈36 hr. Cultured oocytes were directly prepared for host transfer (14) without progesterone maturation. To distinguish groups of oocytes injected with different pools of mRNA from each other and from host eggs as many as four groups (≈40 oocytes per group) were stained with vital dyes of different colors for 15 min before being transferred aseptically into the coelomic cavity of a host female frog that had started to lay her own eggs. Approximately 3 hr after the transfer, the host frog was gently squeezed to help release of its eggs and the injected oocytes. After removing jelly with 45 mM 2-mercaptoethanol, the oocytes and eggs were used for sperm binding assays.

The expression cloning procedure involved injecting mRNAs synthesized from pools of egg cDNA clones into oocytes and then subdividing the pools that positively promoted sperm binding to injected oocytes. This process was repeated until a single clone was isolated. To clone a full-length cDNA, a ≈230-bp fragment from the original cDNA was amplified by PCR, radiolabeled, and used to screen the egg cDNA library by colony hybridization. A full-length cDNA clone was selected and sequenced from both directions.

Northern Blot Analysis. Total RNA (10 μg/lane) was resolved by electrophoresis on a 1% agarose formaldehyde gel. To remove the poly(A) tail, the egg total RNA was hybridized with oligo(dT) and treated with RNase H (25). After electrophoresis, the gel was treated with 50 mM NaOH/10 mM NaCl for 40 min, neutralized in 0.2 M Tris·HCl (pH 7.8) and 18× standard saline citrate (SSC) for 2 × 20 min, blotted onto a Nylon membrane (Hybond, Amersham), and UV-crosslinked. The membrane was prehybridized at 60°C for 6 hr in a buffer containing 1 mM EDTA, 0.5 M Na_2HPO_4 (pH 7.2), 7% SDS, and 1% BSA, and hybridized under the same condition for 24 hr with a radiolabeled, PCR-amplified ≈1-kb cDNA fragment. The blot was washed for 3 × 15 min in 2× SSC, 0.2% SDS, followed by 2 × 15 min in 0.2 × SSC, 0.2% SDS, and exposed to film for 24 hr.

RESULTS

***Xenopus* Oocytes Acquire Sperm Binding Activity After Meiotic Maturation and Oviduct Passage.** Using a quantitative sperm binding assay, we previously showed that sperm do not bind to isolated stage 6 oocytes, nor to coelomic eggs. Rather, sperm bind only to dejellied, oviposited eggs (21). In the current study, we found that when isolated stage 6 oocytes were reintroduced into the coelomic cavity of a host female and allowed to pass through the oviduct without preexposure to progesterone, the recovered oocytes, after removal of their jelly, did not bind sperm (Table 1). This observation suggested that without meiotic maturation passage through the oviduct is not sufficient to induce sperm binding. The results summarized in Table 1 indicate that for the stage 6 oocytes to acquire sperm binding activity, two sequential events are required: (a) maturation of oocytes by exposure to progesterone; and (b) passage of the mature oocytes through oviduct. If either step is omitted, the oocytes do not bind sperm.

As shown by electron microscopic studies, meiotic maturation causes major structural alterations in the oocyte envelope and the plasma membrane (7, 8). We asked if these alterations could contribute to the acquisition of sperm binding capacity during oocyte maturation. Because the proteolytic processing of gp43 in the oviduct is essential for unmasking the putative sperm binding sites in coelomic eggs (8, 16, 17, 21), initially we determined if this processing can occur in immature oocytes. By analyzing the envelope proteins isolated from the immature stage 6 oocytes that have been forced to pass through the oviduct, we found that gp43 was cleaved to gp41 (Fig. 1*A*), a process that normally occurs with coelomic eggs. This result indicates that the proteolytic processing step does not depend on the maturation status of the oocytes. If this is the case, it is possible that the putative sperm-binding site itself on immature oocytes is different from that on mature eggs. To test this hypothesis, we used the polyclonal anti-gp69/64 antibody as a probe to determine if there was a difference in the extent of exposure of gp69/64 in the envelope of mature and immature oocytes after they have passed through the oviduct. Oocyte surface staining experiments with polyclonal anti-gp69/64 antibody and fluorescein isothiocyanate-conjugated secondary antibody (Fig. 1*B*) revealed that after passing through the oviduct and removal of their jelly coats, the progesterone-matured oocytes (Fig. 1*Bc*) exhibited much brighter staining than the immature oocytes (Fig. 1*Bb*). The immature oocytes, which did not show any staining before passing through the oviduct (Fig. 1*Ba*), exhibited a very low, but detectable, amount of staining after oviduct passage (Fig. 1*Bb*). Probably this is due to the cleavage of gp43 and untrastructural alterations that occur to the envelope during oviduct passage. It is noteworthy that the antibody cannot penetrate the intact envelope (Fig. 1*Bd*); it can only recognize and bind the determinants on gp69/64 that are exposed on the egg surface. From these experiments we conclude that,

Table 1. Conditions for oocytes to acquire sperm binding activity

Oocyte manipulations		Sperm binding, bound sperm per oocyte
Meiotic maturation	Oviduct passage	
No	No	5.4 ± 3.5
No	Yes	15.2 ± 6.7
Yes	No	6.6 ± 4.2
Yes	Yes	1,200 ± 139

Stage 6 oocytes that had not been exposed to progesterone or had not passed through the oviduct did not bind sperm (rows 1–3); only after progesterone-induced meiotic maturation followed by passage through the oviduct was sperm-binding observed (row 4). Standard deviations are indicated with $n = 20$ oocytes. See *Materials and Methods* for details on oocyte manipulations.

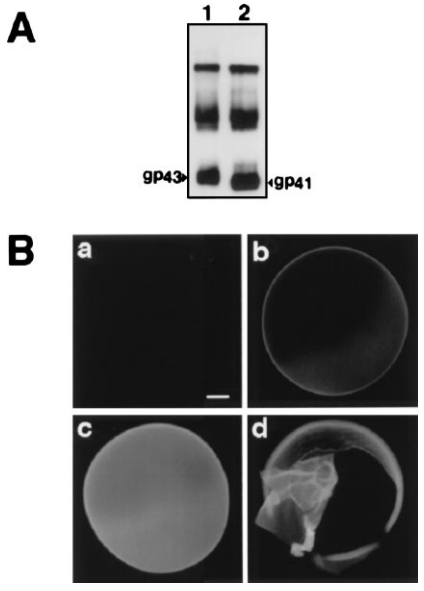

FIG. 1. Effects of meiotic maturation on oocyte surface. (*A*) Western blot of immature oocyte envelope proteins before (lane 1) or after (lane 2) passage of the oocytes through the oviduct. The polyspecific polyclonal anti-"VE" antibody that recognizes gp120/112, 69, 64, 43, and 41 was used. After oviduct passage, gp43 is converted to gp41 (arrows). (*B*) Cell surface staining with specific polyclonal anti-gp69/64 antibody and fluorescein isothiocyanate-labeled secondary antibody of immature oocytes before (*a*) or after (*b*) oviduct passage, and progesterone-matured oocytes after oviduct passage (*c*). In *d* a segment of the VE of an oviposited egg had been torn and laid back after antibody staining and fixation, revealing that the plasma membrane beneath the VE had not been stained. (Bar = 0.2 mm.)

although processing of gp43 to gp41 can occur in the absence of maturation, the maturation process *per se* is necessary for full exposure of functional binding sites.

Injection of Egg Total Poly(A)$^+$ mRNA into Oocytes Promotes Sperm Binding. Having determined that meiotic maturation results in changes at the oocyte surface that affect the accessibility of the sperm binding sites, we assumed that it was likely that this process would require synthesis of new proteins. To test this hypothesis, we asked if injection of total egg poly(A)$^+$ mRNA into stage 6 oocytes could result in acquisition of sperm binding. Up to 60 ng of mRNA isolated from unfertilized eggs was injected into each oocyte, and after *in vitro* culture for 36 hr in the absence of progesterone, the injected oocytes were stained with vital dyes and transferred into the coelomic cavity of a host female that had been induced to lay eggs. After about 3 hr the stained oocytes, enclosed in jelly coat, were oviposited together with the eggs of the host frog. The oocytes then were dejellied and tested for sperm binding activity. The result showed that sperm bound to the oocytes that had been injected with total egg poly(A)$^+$ mRNA, but not to oocytes injected with water or control mRNA. As shown in Fig. 2 the induction of sperm binding was dependent on the amount of total mRNA injected into the oocyte. Compared with the normal level of sperm binding to oviposited eggs (1,300 ± 200), this level was low, although far above control level. However, we think that most of the reduction of sperm binding could be due to mechanical damages caused by the process of removing follicle cells surrounding the oocytes. The oocytes that survived this procedure (35–45%) and showed sperm-binding activity were not mature, because they did not exhibit GVBD and could not be activated by the calcium ionophore A23187. Therefore it is clear that the effects of injection of egg total mRNA were not equivalent to the progesterone-induced maturation process, even though sperm binding ability was induced in the injected oocytes.

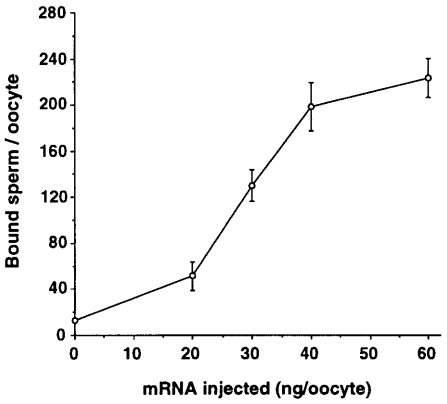

FIG. 2. Injection of total egg poly(A)$^+$ mRNA induces sperm binding to the injected oocytes. Oocytes were injected with 0–60 ng/30 nl of egg poly(A)$^+$ mRNA, incubated in oocyte culture medium for 36 hr, host transferred without exposure to progesterone, and then tested for sperm binding. Bars represent SD with $n = 20$ eggs.

Functional Cloning and Characterization of a *Xenopus* Cdc6 Gene. To identify protein(s) that can promote sperm binding to oocytes, a cDNA library was prepared from total poly(A)$^+$ mRNA isolated from unfertilized eggs. A mixture of capped mRNAs was synthesized from a pool of 10,000 cDNA clones, and 40 ng of this mRNA mixture was injected into the oocytes. After *in vitro* culture and host transfer, the injected oocytes showed an approximately 2.5-fold increase in sperm binding activity compared with control oocytes. With the objective of isolating a single clone(s) that could promote sperm binding, the initial 10,000 clones were subdivided into five pools. Capped mRNAs were made from the five subpools, and each pool was tested for its ability to induce sperm binding to injected oocytes. It has been documented that injection of many different mRNAs or proteins into stage VI oocyte can induce oocyte maturation, although some of the molecules may not be actually used in the endogenous pathway of progesterone-stimulated maturation (26, 27). To avoid recloning known inducers of oocyte maturation, we used a relatively long oocyte culture time (≈36 hr) after injection. A small fraction (<10%) of the mRNA-injected oocytes became mature (as evidenced by the appearance of a white spot in the animal pole) and died during this incubation period. This long incubation step presumably helped to eliminate the mRNA molecules that caused oocyte maturation. Of the five subpools, two were positive in the sperm binding assay, and the pool that induced the highest level of sperm binding was further divided into smaller pools of clones and screened as described above. After a total of 6–7 rounds of subdivision and screening, a single clone was isolated that could induce sperm binding to oocytes injected with its mRNA. Because only the pool that exhibited the highest activity in inducing sperm binding was chosen for further screening, this potentially could result in loss of some other candidates. A ≈230-bp fragment of this cDNA was amplified by PCR, radiolabeled, and used to screen the same egg cDNA phagmid library by colony hybridization. Under conditions of high stringency, five clones of various sizes were positive in duplicate lifts and in a secondary screen. The largest clone had an insert of ≈2.4 kb in length. Capped poly(A)$^+$ mRNA was synthesized from the 2.4-kb insert, and various amounts of this mRNA were injected into groups of stage 6 oocytes. A dose-response relationship was observed between the quantity of the injected mRNA from this clone and the level of sperm binding (Fig. 3). As in the case of injection of total poly(A)$^+$ mRNA, it was observed that oocytes injected with this mRNA could be cultured in oocyte culture medium for 3–4 days without maturation or deterioration.

The nucleotide sequence of this cDNA (GenBank accession no. U76834) was obtained by sequencing in both directions and

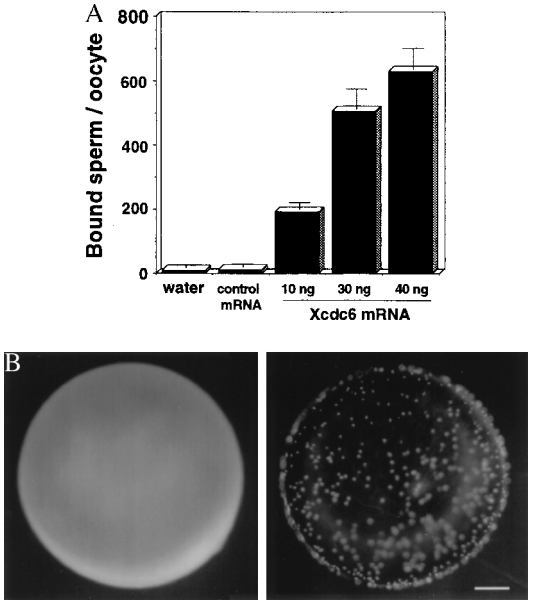

FIG. 3. Injection of Xcdc6 mRNA induces sperm binding to injected oocytes. (*A*) Quantitation of sperm binding to oocytes injected with water, 40 ng of fibronectin mRNA, or 10, 30, and 40 ng of Xcdc6 mRNA. Bars represent SD with $n = 20$ oocytes. (*B*) Fluorescence micrographs showing sperm binding to oocytes injected with 30 ng of Xcdc6 mRNA (*Right*) and to control oocytes injected with water (*Left*). The bright dots on the oocyte surface are sperm heads stained with Hoechst 33342. (Bar = 0.2 mm.)

the largest predicted ORF contained 554-aa residues (Fig. 4). The deduced ORF was believed to be full length because stop codons were found in all three different reading frames immediately flanking the start and the stop codons that defined the ORF. The M_r of the bacterially expressed Xcdc6 protein was approximately 61–62 kDa as determined by SDS/PAGE (data not shown), which is consistent with the size of the deduced ORF. A BLASTP search of the GenBank indicated that the ORF was identical to that recently reported by Coleman *et al.* (28), and it encoded the 61-kDa *Xenopus* Cdc6 protein. As shown in Fig. 4, in addition to a ATP/GTP binding site and two nuclear localization signal sequences, Xcdc6 protein has a putative leucine zipper motif with the sequence **L**PNSRMV**L**IGIANA**L**DLTDRI**L** (29) and five consensus phosphorylation sites (S/T-P-X-Z, where X is any amino acid and Z is a basic amino acid) for the $p34^{cdc2}$/cyclin B protein kinase (30). It also has a potential N-linked glycosylation site. The leucine zipper motif and the first three $p34^{cdc2}$ kinase phosphorylation sites were found to be conserved in the human Cdc6 protein (31).

The cloned Xcdc6 cDNA includes a 5′-untranslated region (UTR) of 125 nucleotides and a 3′-UTR of 532 nucleotides that have not been reported previously. A long poly(A) tail was found attached to the 3′-UTR of this clone. As shown in Fig. 4, multiple copies of two cis-acting sequences that regulate polyadenylation of mRNA during oocyte maturation were found in the 3′-UTR. The two sequences include the cytoplasmic polyadenylation element (CPE) with the consensus sequence UUUUUAU (32, 33) and the hexanucleotide polyadenylation signal AAUAAA, which is also necessary for nuclear pre-mRNA polyadenylation (33). Another type of CPE, with a minimum of dodecauridine that is used to direct polyadenylation during embryogenesis (34, 35) also was found in the 3′-UTR of Xcdc6 ($U_6GCU_{12}G$).

The presence of these sequences suggested that the Xcdc6 mRNA may be subjected to cytoplasmic polyadenylation during oocyte maturation and development. Northern blot analysis agreed with this hypothesis. The Xcdc6 transcripts (≈2.4 kb) were found in all the early developmental stages tested, including the stage 6 oocyte, unfertilized egg, fertilized egg (0.5 hr after fertilization), and dividing embryos at two-cell and eight-cell

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ATTTTGCCGCGCTGGGGGTCAGTGTGCGGCGACGTTTAGAGCAGAAGAAGCAGCGGTGGC   60
AGCAGTGAGGGCAGCGCGTTTTTATTTCTGACTGGAATCCGCCACAGCCTCGCTGACAAG  120
CAGCCATGCCAAGCACCAGGTCTCGGTCTCAAAGCTCCATTCAGTTTCCCAAGAAAAAGA  180
      M  P  S  T  R  S  R  S  Q  S  S  I  Q  F  P  K  K  K     18
CTTCTCAGACGCTCGCCAAAGAGGTCTCACGTGCAAAGAGCAAGTCTGAGATCTGCTCCT  240
T  S  Q  T  L  A  K  E  V  S  R  A  K  S  K  S  E  I  C  S    38
                NLS                    NLS
CTGTCTCCCTCCCGCTCTCTCCACTTCCCAAAGAGCTTCCCCTCAGTCCACGCAAACGGC  300
S  V  S  L  P  L  S  P  L  P  K  E  L  P  L  S  P  R  K  R    58
TCGGTGATGACAATCGTTGCAACATTCCTCCGACATTAAGCTGCTCCCCACCCAAGCAGT  360
L  G  D  D  N  R  C  N  I  P  P  T  L  S  C  S  P  P  K  Q    78
CTCGCAAAGAGACTGGCCAGCCAACCACCCCTAAGGGGGGCCGTTTACTTTTTGATGAGA  420
S  R  K  E  T  G  Q  P  T  T  P  K  G  G  R  L  L  F  D  E    98
ACCAGGCTGCAGCAGCGACACCACTATCCCCCCTCAAGAAGCTACAGGATCCTTATCAGC  480
N  Q  A  A  A  A  T  P  L  S  P  L  K  K  L  Q  D  P  Y  Q   118
TGTCCCCTGTGAGAAAGGGGGCAAGAGACCCCACCCAGCTCTCGTAAGCAAAGGAACAGTG  540
L  S  P  V  R  K  G  Q  E  T  P  P  S  S  R  K  Q  R  N  S   138
                                  NLS
TGGGGGTCCAGCTATTTAAACAGGAGGGCTCCTGCTATCAGAAGGCTAAGCACGCTTTGA  600
V  G  V  Q  L  F  K  Q  E  G  S  C  Y  Q  K  A  K  H  A  L   158
ATACGGCTATACCCGAGCGCCTGTTGGCTCGTGAGAGTGAGACTGCATTTATCAAGACCT  660
N  T  A  I  P  E  R  L  L  A  R  E  S  E  T  A  F  I  K  T   178
TCCTGACAAGTCATGTTTCTGCTAGGAAAGCCGGGAGCCTTTACATATCTGGTGCTCCTG  720
F  L  T  S  H  V  S  A  R  K  A  G  S  L  Y  I  S  G  A  P   198
                                                  ATP/GTP-
GAACTGGCAAAACTGCGTGCTTGAATAAGCTGCTGCAGGAGAGCAAGGATGATCTCAAGC  780
G  T  G  K  T  A  C  L  N  K  L  L  Q  E  S  K  D  D  L  K   218
Binding
AGTGCAAGACTGTTTACATCAACTGCATGTCATTACGCAGCTCCCAGGCAGTGTTTCCGG  840
Q  C  K  T  V  Y  I  N  C  M  S  L  R  S  S  Q  A  V  F  P   238
CAATAGCTGAAGAAATCTCTGGGGGGAAATCTTCCCTTGCTGCAAAAGACATGGTCAGGA  900
A  I  A  E  E  I  S  G  G  K  S  S  L  A  A  K  D  M  V  R   258
ATTTGGAAAAGCTGGTGACTTCAAAGGGTCCAATCATCTTGTTGGTGTTGGATGAGATGG  960
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CAAATTCCAGGATGGTTCTAATTGGCATTGCTAATGCGTTGGATTTGACAGACCGTATTT 1080
P  N  S  R  M  V  L  I  G  I  A  N  A  L  D  L  T  D  R  I   318
                  LEU-ZIP                    LEU-ZIP
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L  P  R  L  Q  A  R  P  Q  C  K  P  Q  L  L  N  F  S  P  Y   338
CAAAGGATCAGATTGCTACCATTCTGCAAGACAGACTAAATCAGGTGTCAGGTGATCAAG 1200
T  K  D  Q  I  A  T  I  L  Q  D  R  L  N  Q  V  S  G  D  Q   358
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V  L  D  N  A  A  I  Q  F  C  A  R  K  I  S  A  V  S  G  D   378
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N  P  V  P  K  K  V  S  L  P  H  I  S  R  V  L  S  D  V  Y   438
GAGACAAGATGGCAAGCAATGGGGGGTCCAGCGACAGTTTTCCTTTACAGCAGAAACTGC 1500
G  D  K  M  A  S  N  G  G  S  S  D  S  F  P  L  Q  Q  K  L   458
TGGTCTGTGCCCTGCTGCTAATAACTCGCCAAAGCAAGATCAAGGAGGTCACGCTTGGCA 1560
L  V  C  A  L  L  L  I  T  R  Q  S  K  I  K  E  V  T  L  G   478
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K  V  H  E  A  Y  S  K  V  C  R  K  Q  Q  V  P  G  V  G  Q   498
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S  E  C  L  S  L  C  Q  L  L  E  T  R  G  I  L  G  L  K  K   518
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A  F  K  D  K  L  L  I  G  N  V  L  N  S  G  I  *            554
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AAGGAGGGTGAAAAATCACAGGCCTTTTAAACACAGGCAAACAATTACTTTTCAATATTT 1980
GGTTGTTTTCTACTGTTCAGTACAGTCATAAAGATTGACCTGTTTTGTAAAGTATCTGCA 2040
CGTGACATAACTAGTTTCTGCTGTAAATAAATAGTCTGGTTTTAATTTGCTGTCGTGT 2100
GCAGCAGATGTTAATCTTTAAGTATCTGTAAGGTACGGCCCTGTTTGTTTTTTTTTATAG 2160
ACAAGGTGTTTTTTATCCCCTGGATTAGACTTGCGTTTTATTCAAGAATCCACTTCTGTTT 2220
TAATAAACCATTAAACCATTTTTTGCTTTTTTTTTTTTTGCTTTCTATTGTGAAGGGATAT 2280
GGCCAACTCTGATGAATAAAACACATCAGTTTGTTTGAGGTGGAAAAAAAAAAAAAAAAA 2340
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 2400
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FIG. 4. Nucleotide sequence of Xcdc6 cDNA and deduced amino acid sequence. In the 3′-UTR of the nucleotide sequence, the CPE sequence (TTTTTAT) and the poly(A) signal sequence (AATAAA) are indicated. The embryonic-type CPE sequence is boxed. In the amino acid sequence, the two nuclear localization signals (NLS), leucine zipper motif, and ATP/GTP binding domains are underlined and labeled. The N-linked glycosylation site is double underlined. The five $p34^{cdc2}$/cyclin B consensus sites are boxed.

stages (Fig. 5). The level of the mRNA remains essentially constant. However, the size of the transcripts increases during oocyte maturation. When the poly(A) tails of the total egg mRNA mixture is removed by hybridization with oligo(dT) and RNase H treatment, the Xcdc6 transcript (Fig. 5, lane 0) was reduced to approximately the same size as the cloned cDNA without its poly(A) tail, indicating that the cloned Xcdc6 cDNA is likely to be full length.

Injection of Xcdc6 mRNA Does Not Block Normal Progesterone-Induced Oocyte Maturation nor Fertilization. To determine if injection of Xcdc6 mRNA has any effects on the normal progesterone-induced oocyte maturation process or on fertilization or embryonic development, we incubated the oocytes that had been injected with 15 ng of Xcdc6 mRNA in OR2 medium containing 3 μM progesterone. After 6–8 hr of incubation, more than 85% (n = 40) of the oocytes became mature as scored by their ability to undergo GVBD. As compared with noninjected control oocytes, no significant increase or decrease in time to reach GVBD was noted. These mature oocytes then were introduced into the coelomic cavity of a host frog and allowed to pass through the oviduct. The recovered mature oocytes immediately were mixed with sperm and tested for fertilization efficiency. It was found that the fertilization rate of the mature oocytes that had been injected with Xcdc6 mRNA was close to control oocytes injected with water, 60% vs. 70% with n = 30 for each group. This result suggested that although injection of exogenous Xcdc6 mRNA alone induced changes that resulted in sperm binding, these changes did not have adverse effects on the normal process of progesterone-induced oocyte maturation or fertilization. However, it was found that the embryos resulted from fertilization of the progesterone-matured oocytes that had been injected with Xcdc6 mRNA did not develop normally. They typically showed severe defects in gastrulation and greater than 80% of the embryos did not survive beyond the gastrulation stage (data not shown).

DISCUSSION

The molecular mechanism of fertilization in *X. laevis* has just begun to be elucidated, with many important questions remaining to be answered. In the current study, we investigated the development of fertilizability in *Xenopus* oocytes and focused on one aspect of this process: the acquisition of sperm-binding ability. It was found that for the immature stage 6 oocyte to develop the ability to bind sperm at fertilization, two steps are essential. First, the oocyte has to become meiotically mature by exposure to progesterone; second, the mature oocyte must pass through the oviduct where the oocyte surface is modified and sperm binding sites on the VE are unmasked. If either of the two steps is omitted, the oocyte cannot bind sperm. Ultrastructural studies using special electron microscopic techniques have revealed significant differences between the surface of the immature stage 6 oocyte and that of the mature coelomic egg (7, 8). Based on those studies, it is believed that the plasma membrane of stage 6 oocytes is highly folded, with long microvilli extending into the oocyte envelope, which consists of tight bundles of long, thick protein filaments. After meiotic maturation, the plasma membrane flattens and the microvilli become shortened, but more ordered. At this stage, the entire CE is elevated above the microvilli tips, but probably still makes contacts with them. In addition, the filaments in the CE become more loosely bundled, creating large spaces in the envelope.

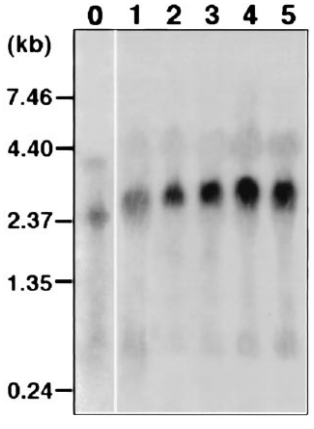

FIG. 5. Northern blot analysis of developmental expression of Xcdc6 mRNA. RNA samples were isolated from stage 6 oocyte (lane 1), unfertilized egg (lane 2), fertilized egg (lane 3), and embryos at two-cell (lane 4) and eight-cell (lane 5) stages. RNA without poly(A) tail was prepared by digesting total egg RNA with RNase H in the presence of poly(dT)$_{12-18}$ (lane 0). Total mRNA (10 μg) was loaded in each lane, and equivalent loading was confirmed by staining of ribosomal RNA subunits with ethidium bromide (data not shown).

To understand how these ultrastructural changes may affect the sperm-binding property of the oocyte surface, we introduced stage 6 oocytes either before or after incubation with progesterone to induce meiotic maturation into the coelomic cavity of the frog and allowed them to pass through the oviduct. By analyzing the envelope proteins isolated from these two groups of oocytes, we found that the proteolytic processing of gp43, a major component of the oocyte envelope, occurred in both mature and immature oocytes. Based on previous studies (8, 16, 17), we believe that cleavage of gp43 to gp41 is a prerequisite to subsequent formation of functional sperm binding site consisting of the VE glycoprotein, gp69/64, and possibly other components (21). By comparing the intensity of polyclonal anti-gp69/64 antibody staining on the surface of the mature and immature oocytes after both had passed through the oviduct, we searched for changes in the sperm binding sites that might affect the conformation or accessibility of gp69/64. The result of this experiment showed that the antibodies stained the surface of the mature oocytes much more intensely than that of the immature oocytes. This result suggests that independent to the processing of gp43, additional changes in the sperm binding sites caused by meiotic maturation process also contribute to the acquisition of sperm binding ability. These changes may involve either ultrastructural alterations or addition of new components. However, we have not yet been able to detect compositional changes in the envelope or in the plasma membrane caused by oocyte maturation using detection methods not targeted to any specific component (data not shown). In any case what is clear from these studies is that two stages are involved in formation and exposure of functional sperm binding sites. In the first stage, meiotic maturation results in unknown changes that are required for subsequent binding of sperm by gp69/64; in the second stage, the gp69/64 molecules are exposed in functional form due to proteolytic processing of gp43 to gp41 and corresponding ultrastructural changes in the envelope.

To determine if new protein synthesis might be essential in the acquisition of sperm binding ability, we injected egg poly(A)$^+$ mRNA into oocytes and found that the injected oocytes showed higher levels of sperm binding than control oocytes after passage through the oviduct. The rationale of this experiment was that by introducing purified total poly(A)$^+$ mRNA from unfertilized egg into stage 6 oocyte, we might mimic the unmasking and translational activation processes that occur to the maternal mRNAs during oocyte maturation, because the injected egg mRNAs are devoid of RNA binding or masking factors and are likely to have undergone cytoplasmic polyadenylation. Although this approach is only an approximation of what happens during oocyte maturation and could produce nonspecific effects, the result confirmed that expression of certain mRNAs present in the unfertilized egg can induce changes that result in acquisition of sperm binding.

In an effort to identify a protein(s) that might be responsible for acquisition of sperm binding, we designed an expression cloning strategy and used it to screen an egg cDNA library. The

criteria used in this screen was that the injected mRNA should promote sperm binding while not causing complete oocyte maturation. A 36-hr *in vitro* oocyte culture step was included in the procedure to reduce the chance of cloning factors that can induce oocyte maturation. One positive clone resulting from this screen was found to encode a protein related to the yeast Cdc6/Cdc18 proteins. The deduced amino acid sequence later was confirmed to be identical to the recently identified *Xenopus* Cdc6 protein (28).

Based on the current understanding of the mechanism of Cdc6 action, it is not yet clear how this protein is involved in the induction of sperm binding. The Cdc6 protein in yeast *Saccharomyces cerevisiae* and its related protein Cdc18 in *Schizosaccharomyces pombe* both are required for initiation of DNA replication and for ensuring that DNA replication is complete before mitosis (36–44). Lack of Cdc6/18 causes cells to enter mitosis without DNA replication. On the other hand, overexpression of Cdc18 results in repeated rounds of DNA replication in the absence of mitosis. Studies on the recently cloned *Xenopus* and human Cdc6 homologs indicate that these proteins also play an essential role in the initiation of DNA replication (28, 31). Currently, it is believed that interactions of Cdc6/18 with a variety of other protein complexes, such as the origin recognition complex, the minichromosome maintenance protein complex, and cyclin-dependent kinases are important in coordinating chromosome replication and mitotic cell cycle control (reviewed in ref. 45). However, little is known about the regulation and mechanism of Xcdc6 function during oocyte meiotic maturation.

Although we are still not certain if the induction of sperm binding by Xcdc6 represents a specific physiological process that actually takes place during normal *Xenopus* oocyte maturation, several possibilities can be considered. One is that the observed effect could be the result of interaction between Xcdc6 protein with the maturation promoting factor (MPF). The Xcdc6 protein contains five $p34^{cdc2}$/cyclin B protein kinase consensus sites, and three of them are conserved in the human Cdc6 protein. Yeast Cdc6 protein also has five such sites and recently has been demonstrated to be an *in vitro* substrate of the Cdc28 kinase (homolog to Cdc2 in fission yeast). In addition, Cdc6 can inhibit Cdc28 histone H1 kinase activity and can be coimmunoprecipitated with Cdc28 (46). Cdc18 also interacts with cyclin-dependent kinase in fission yeast (47). In *Xenopus*, the MPF (also called the mitosis and meiosis promoting factor) activity is essential for entry into meiosis and mitosis (48, 49). If Xcdc6 physically interacts with MPF, we suggest that overexpression of Xcdc6 protein somehow may affect a subset of MPF activities that turn on certain cell cycle events and lead to alterations on the cell surface that affect sperm binding.

Alternatively, microinjection of Xcdc6 mRNA with a long poly(A) tail may mimic the effect of translational activation of the message during oocyte maturation. The maternal Xcdc6 mRNA was found to undergo cytoplasmic polyadenylation during oocyte maturation. The presence of multiple copies of the maturation-type CPE sequence (UUUUUAU) and the conserved polyadenylation signal (AAUAAA) in the 3′-UTR also predict that Xcdc6 mRNA can be translationally activated during meiotic maturation. Because Xcdc6 protein presumably can interact with a variety of other factors important in chromosome replication and cell cycle control (28, 45), the elevated Xcdc6 mRNA and protein levels in the injected oocytes could perturb the complex cell-cycle control system, which might result in initiation of events that normally occur during meiotic maturation, such as new protein synthesis required for appearance of a functional sperm receptor.

It has been observed that in fission yeast overexpression of Cdc18 triggers multiple aberrant rounds of DNA replication (41, 44) and that constitutive expression of *S. cerevisiae* Cdc6p can delay entry into mitosis (38). However, in *Xenopus* oocytes we have not found any evidence that overexpression of Xcdc6 can trigger DNA replication (data not shown). Similarly, *in vitro* studies have shown that addition of His6-Xcdc6 protein to egg extracts does not stimulate replication above normal levels (28). We also observed that injection of Xcdc6 mRNA into oocytes that then are subjected to host transfer does not seem to affect normal maturation of these oocytes or subsequent fertilization. However, it may be noteworthy that the embryos developed from the oocytes injected with Xcdc6 mRNA showed severe defects during early embryogenesis. This could reflect a difference in the ability of eggs and embryonic cells to cope with high concentrations of the Xcdc6 protein. Analysis of the effect of overexpression of Xcdc6 in frog oocytes and embryos remains a subject for future studies.

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