Development 124, 493-503 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV4771

Spindlin, a major maternal transcript expressed in the mouse during the transition from oocyte to embryo

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SUMMARY

Timely translation of maternal transcripts and post-translational modification of their gene products control the initial development of preimplantation-stage embryos. We have isolated and characterized a gene encoding a stage-specific embryonic protein. This novel gene, spindlin (*Spin*), is an abundant maternal transcript present in the unfertilized egg and 2-cell, but not 8-cell, stage embryo. *Spin* exhibits high homology to a multicopy gene, Y-linked spermiogenesis-specific transcript (*Ssty*), and together they form a new gene family expressed during gametogenesis. We find that spindlin associates with the meiotic spindle and is modified by phosphorylation in a cell-cycle-dependent fashion. Furthermore, it comigrates with the previously described 30×10^3 M_r metaphase complex which is post-translationally modified during the first mitotic cell cycle. Our data suggest that spindlin plays a role in cell-cycle regulation during the transition from gamete to embryo.

Key words: spindlin, maternal transcript, 30×10^3 M_r complex, meiotic cell cycle, spindle, oocyte, preimplantation embryo, mouse

INTRODUCTION

Successful reproduction in mammals requires proper maturation of the female gamete, during which process the oocyte progresses through the meiotic cell cycles and arrests in meiotic metaphase II at ovulation (reviewed in Eppig, 1993). Fertilization initiates the cascade of signal transduction resulting in completion of the oocyte meiotic cell cycle and in activation of the mitotic cell cycle. Although the morphological events during the developmental period of transition from gamete to embryo are well described, little is known about the molecules that drive these events. A network of protein phosphorylation and dephosphorylation appears to regulate the progression of the meiotic cell cycle. A complex of cyclin B and $p34^{cdc2}$ kinase, a universal M-phase promoting factor (MPF), triggers the G_2/M_I transition and displays cyclic activity during the meiotic cell cycle. Transcription in the oocyte ceases upon meiotic maturation when the chromosomes of the oocyte are completely condensed (Knowland and Graham, 1972; Moore, 1975), and does not reinitiate until the embryonic genome is activated in the 2-cell-stage embryo (Schultz, 1993). Thus, meiotic maturation of the full-grown oocyte, maintenance of the unfertilized egg and development of the zygote and cleavage-stage embryo depend in part on the timely translation of maternal mRNAs. Upon transport into the cytoplasm, some mRNAs synthesized in oocytes are deadenylated and stored as dormant messages without translation (Huarte et al., 1992; Bachvarova, 1992). Translational activation of these dormant mRNAs is initiated by cytoplasmic polyadenylation, which requires the presence of two *cis*-acting elements in the 3′ untranslated region (UTR) of the mRNAs: a nuclear polyadenylation signal and a UA-rich cytoplasmic polyadenylation element (CPE) (Fox et al., 1989; McGrew and Richter, 1990; Salles et al., 1992). Activation of the stored maternal messages appears to occur at oocyte meiotic maturation and after fertilization (Fox et al., 1989; McGrew et al., 1989; Simon et al., 1992; Simon and Richter, 1994).

Biochemical studies have demonstrated changes in protein synthetic patterns during mouse oocyte maturation (Globus and Stein, 1976; Schultz and Wassarman, 1977) and also after fertilization of the egg (Levinson et al., 1978; Howe and Solter, 1979; Van Blerkom, 1981; Cullen et al., 1980; Cascio and Wassarman, 1982; Pratt et al., 1983; Howlett and Bolton, 1985, Latham et al., 1991). Since these stages are transcriptionally inert, the changes in protein pattern may reflect neo-translation of stored maternal transcripts or post-translational modification of their products. These molecules may be essential for the developmental processes during this time of transition from gamete to embryo. However, due to the difficulty of obtaining a sufficient quantity of oocytes and embryos for molecular studies, identification of such molecules critical to preimplantation embryogenesis has been challenging. To explore the molecular basis of the development during this transition period, we constructed cDNA libraries from mouse eggs and preimplantation embryos, subtracting them from each other to obtain probes for genes expressed in a stage-specific fashion (Rothstein et al., 1992). We report here the isolation and characterization of a gene, *Spin*, which encodes a stage-specific embryonic protein designated spindlin. In the ovary, expression

of the *Spin* transcript is restricted to oocytes and its peptide sequence has high homology to *Ssty*, a Y-linked spermiogenesis-specific transcript, throughout the entire open reading frame. Using antibody raised to spindlin, we demonstrate that this protein is phosphorylated in a cell-cycle-dependent fashion and associated with the spindle during the meiotic cell cycle. We find that *Spin* encodes a protein complex, previously shown to be modified in a cell-cycle-dependent manner in the egg and zygote (Howlett, 1986).

MATERIALS AND METHODS

cDNA isolation and sequencing

A 0.9 kb stage-specific embryonic cDNA clone (SSEC) P was obtained from a subtraction library produced by subtracting a 2-cell-stage mouse embryo cDNA library with at least a 100-fold excess of the 8-cell-stage cDNA library and a 5-fold excess of the egg cDNA library (Rothstein et al., 1992, 1993). The 0.9 kb cDNA was used as a probe to obtain the 4.1 kb full-length clone from the 2-cell-stage primary cDNA library. Smaller 1.7 kb and 0.8 kb SSEC P cDNA clones were also isolated from the same 2-cell primary cDNA library using a 0.7 kb *Bam*HI-*Hin*dIII fragment of the 4.1 kb clone containing the open reading frame sequence. For sequencing, the full-length 4.1 kb clone was cleaved with restriction enzymes and the fragments were subcloned into pBluescript SK(+) (Stratagene) and sequenced using the Sequenase kit (US Biochemical) according to the manufacturer's instructions.

Oocyte and fertilized egg collection and culture

Oocytes and fertilized eggs were collected from B6D2 F_1/J mice (The Jackson Laboratory, Bar Harbor, ME) in M2 or MEM medium supplemented with 4 mg/ml bovine serum albumin (BSA; Hogan et al., 1986) and cultured at 37°C in 100 μl drops of M16 or MEM/BSA medium under mineral oil in a 5% O_2, 5% CO_2 and N_2 atmosphere in a Billups-Rothenberg incubator chamber. Full-grown germinal vesicle-intact oocytes were obtained by needle-puncture of ovaries from 3-week-old mice, injected 46-48 hours earlier with pregnant mare serum gonadotrophin (PMSG; Sigma). Ovulated oocytes were collected from oviducts of mice superovulated with PMSG and human chorionic gonadotropin (hCG; Sigma), by tearing the ampulla of the oviduct and collecting the cumulus mass (Hogan et al., 1986). Surrounding cumulus cells were removed from the cumulus masses by treatment with 500 μg/ml hyaluronidase. Fertilized eggs were collected by delayed mating: superovulated female mice were individually caged with experienced males for 1 hour, checked for copulatory plugs and fertilized eggs were collected from the plugged females by flushing the oviduct with M2 or MEM/BSA medium. For nocodazol treatment, 14 hour-postfertilized eggs were cultured overnight in M2 or MEM/BSA supplemented with 10 μM nocodazol (Sigma). Then the metaphase-arrested zygotes were radiolabeled with [^{35}S]methionine as described below.

Northern blot analysis and RT-PCR

Oocyte RNA was prepared by the method of Huarte et al. (1987) using guanidium thiocyanate and carrier tRNA. After transfer of RNA to MSI nylon membranes (Micron Separations, Inc.), northern blot analysis was performed by hybridizing [^{32}P]dCTP-labeled probes in a hybridization solution containing 50% formamide, 5× Denhardt's solution, 5× SSC, 0.2% SDS, 100 μg/ml denatured salmon sperm DNA and 10% dextran sulfate at 42°C overnight. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed as described elsewhere (Ohsugi et al., 1996). Briefly, the RT reaction was conducted on 100 eggs or embryos in 20 μl volume, then 1 μl of the RT reaction was used for PCR in the presence of 1 μCi ^{32}PdCTP. PCR conditions were 30 cycles of 30 seconds at 94°C, 30 seconds at 60°C, and 30 seconds at 72°C. Primers were 5′-gtgggccgctctaggcaccaa-3′ and 5′-ctctttgatgtcacgcacgattc-3′ for β-actin, and 5′-catcctccatggcctctgcgtcaag-3′ and 5′-gtacatgtacaagacagggt-3′ for spindlin.

In situ hybridization

Mouse ovaries were fixed by overnight incubation in 4% paraformaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin. In situ hybridization to ovarian cross-sections was performed as described (Kessel and Gruss, 1991). Briefly, the 0.7 kb *Bam*HI-*Hin*dIII open reading frame cDNA sequence was subcloned into the pBluescript SK(+) vector (Stratagene), linearized with a *Bam*HI digestion and transcribed as the antisense riboprobe using T7 RNA polymerase (Stratagene) in the presence of 2 mCi/ml [^{35}S]UTP (specific activity >1,000 Ci/mmol; Amersham). Approximately 10^8 cts/minute/ml of ^{35}S-radiolabeled riboprobe was used for the hybridization for 16 hours at 50°C. Sections were exposed for 10 days at 4°C to Kodak NTB-2 emulsion film for autoradiography. After development in Kodak D-19 solution, the slide was counterstained briefly with hematoxylin and examined with a Zeiss Axiophot microscope.

Preparation of antibody and immunohistochemistry

A 0.7 kb *Bam*HI-*Hin*dIII coding sequence (128 nt-840 nt) was cloned into pTrcHis vector containing six histidine residues (Invitrogen) and expressed in the *E. coli* TOP10 strain according to the manufacturer's instructions. The expressed protein was purified through a Zn^{2+} affinity column using denaturing conditions. Polyclonal antibodies were raised in a rabbit by injecting approximately 100 μg of purified protein and boosting with the same amount at 2, 3 and 7 weeks (CoCalico Inc.).

Mouse ovaries were fixed by overnight incubation in Bouin's solution (85 parts saturated picric acid, 10 parts formaldehyde and 5 parts glacial acetic acid) and embedded in paraffin. The sectioned slides were incubated for 30 minutes in 3% BSA/PBS, rinsed with 0.25% BSA/PBS and incubated for 60 minutes at room temperature in a humidified chamber with normal rabbit serum or rabbit antiserum to SSEC P diluted 400-fold in 1% BSA/PBS. Slides were then incubated for 40 minutes with biotinylated goat anti-rabbit IgG (Sigma ExtrAvidin kit) diluted 20-fold in 1% BSA/PBS and for 40 minutes in peroxidase-conjugated avidin diluted 20-fold in 1% BSA/PBS. For visualization of the bound antibody, 3,3′-diaminobenzidine (Sigma Fast DAB tablet) was used as a substrate for peroxidase. The color reaction was stopped by rinsing the slides in water. Sections were counterstained with hematoxylin.

Whole-mount immunolocalization

Ovulated oocytes obtained from superovulated mice were washed through several drops of 0.4% polyvinyl pyrrolidone (PVP)/PBS, fixed with Bouin's solution for 30 minutes at room temperature and washed twice with PVP/PBS. After permeabilization in 0. 5% Triton X-100/PBS for 10 minutes, the processed oocytes were incubated in 1% fetal calf serum/PBS for at least 1 hour at 4°C for blocking and for 2 hours at 37°C in a 50 μl drop of anti-SSEC P antibody serum diluted 200-fold with 1% BSA/PBS. Oocytes were washed twice in an excess volume of 1% BSA/PBS for 15 minutes at room temperature with constant shaking, incubated with secondary antibody (goat anti-rabbit) conjugated with Cy3 for 1 hour at 37°C and washed as before. A Zeiss confocal microscope was used to examine the whole-mount immunostained oocytes.

Radiolabeling, immunoprecipitation and protein gel electrophoresis

Oocytes and fertilized eggs were incubated in M16 medium containing 1 mCi/ml L-[^{35}S]methionine (specific activity >1,000 Ci/mmol; Amersham) for 3 hours (2 hours for fertilized eggs) or in phosphate-free modified DMEM medium containing 0.5 mCi/ml [^{32}P]orthophos-

phate (New England Nuclear) for 3 hours. Labeled oocytes and fertilized eggs were lysed in 100 μl immunoprecipitation (IP) buffer containing protease inhibitors (Hampl and Eppig, 1995). The lysate was precleared with protein A-Sepharose CL-4B (Sigma) preincubated with 25 μg normal rabbit immunoglobulin and immunoprecipitated with protein A-Sepharose beads preincubated with 25 μg anti-SSEC P immunoglobulin by incubation for 1 hour at 4°C. For polyacrylamide gel electrophoresis, the Sepharose beads were boiled in SDS buffer and the elutes were run on a 12% polyacrylamide gel. For two-dimensional gel electrophoresis, approximately 100 L-[^{35}S]methionine-labeled oocytes were lysed in 100 μl IP buffer, immunoprecipitated and eluted from protein A-Sepharose-beads by incubation in urea sample buffer for 20 minutes at 42°C. Either Rainbow Markers (Amersham) or MW-SDS-70L (Sigma) was used as protein molecular weight standards. The gel was dried and exposed on Kodak Biomax film for autoradiography.

RESULTS

Cloning and sequence analysis of SSEC P

To identify genes that encode molecules expressed in a stage-specific manner, we isolated several cDNA clones from a 2-cell-stage embryo subtraction library (Rothstein et al., 1992; Hwang et al., 1996). One of these stage-specific embryonic cDNA clones, SSEC P, is a novel and very abundant maternal transcript. In fact, 0.35% of the total clones in the 2-cell-stage cDNA library are represented by SSEC P, whereas only 0.08% are β-actin. In contrast, only 1 of 2×10^5 cDNA clones tested in the 8-cell-stage cDNA library hybridized to an SSEC P probe. A full-length SSEC P cDNA clone was obtained by screening the primary 2-cell-stage cDNA library with the original 0.9 kb

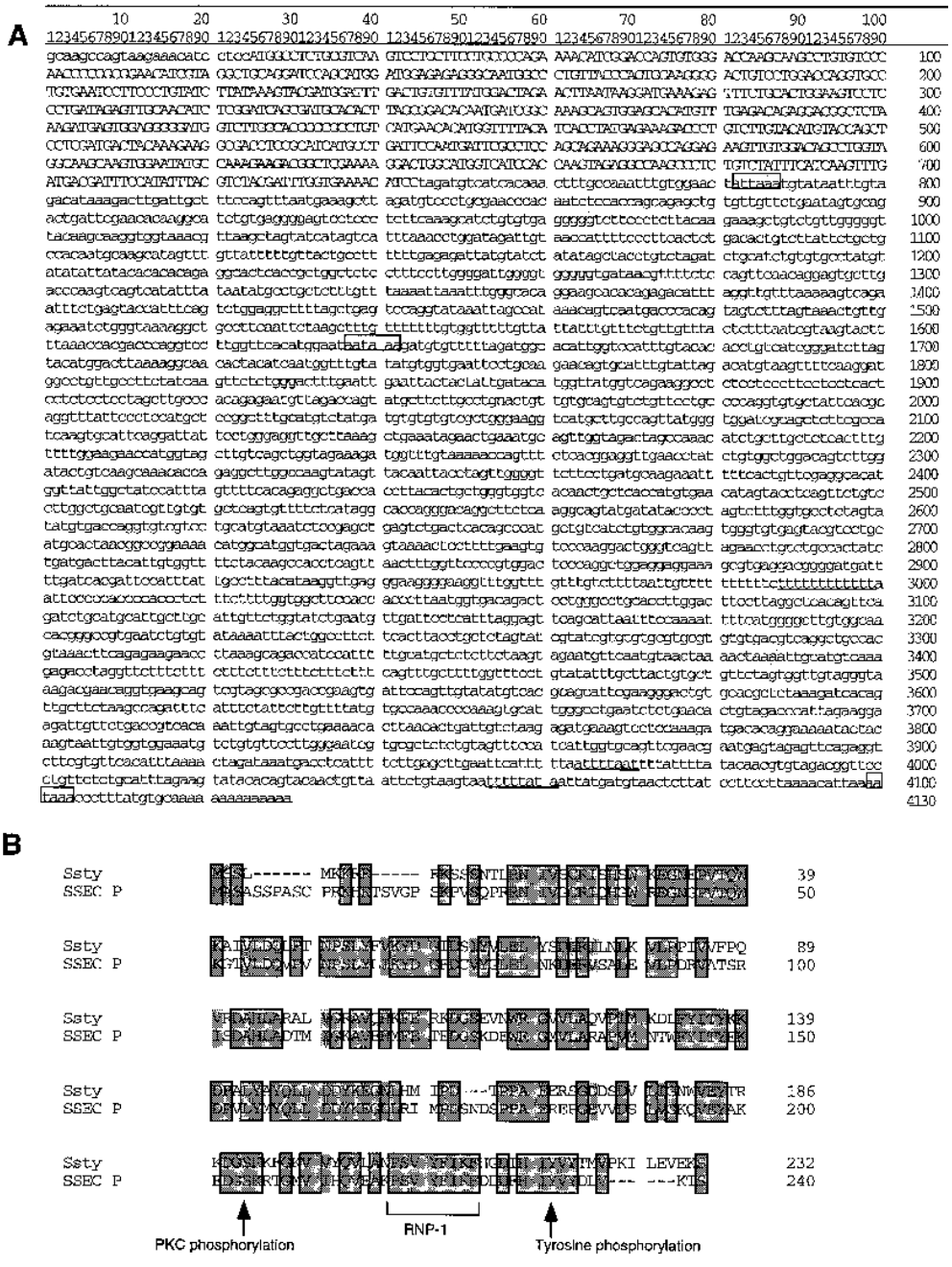

Fig. 1. SSEC P cDNA sequence and comparison of its peptide sequence to a testis homolog. (A) The full-length sequence of the 4.1 kb SSEC P cDNA clone, containing a 720 nt open reading frame (capitalized) encoding 240 amino acids starting with an ATG codon at 25 nt and ending with TAG at 747 nt. The three polyadenylation signals, AUUAAA at 782 nt, AAUAAA at 1,637 nt and AAUAAA at 4,099 nt, are boxed. Three cytoplasmic polyadenylation sequences, dodecauridine (TTTTTTTTTTTT) at 2,988 nt, AUUUUAAU at 3,964 nt, and UUUUUAUA at 4,054 nt, are underlined. (B) SSEC P peptide sequence compared to that of a testis homolog, *Ssty*. The amino acid residues identified between SSEC P and the homolog are boxed and those showing the similarity are shaded. The polypeptide shown in this panel is the entire sequence of SSEC P deduced from the open reading frame of the cDNA. Arrows indicates two potential phosphorylation sites conserved in this gene family, a protein kinase C phosphorylation site at amino acid 203 and a tyrosine phosphorylation site at amino acid 234. The RNP-1 motif is also indicated.

subtraction library probe. The 4.1 kb full-length cDNA contains a 720 base potential open reading frame, encoding 240 amino acids, and an unusually long 3′ UTR of 3371 bases (Fig. 1A). This long 3′UTR contains three conserved cytoplasmic polyadenylation elements (CPEs) (underlined in Fig. 1A), which facilitate elongation of the poly(A) tail and translation of maternal transcripts stored in the cytoplasm at particular times during development (Fox et al., 1989; McGrew and Richter, 1990). The first sequence, UUUUUAUA (4,054 nt), is found 37 nt upstream of the nuclear polyadenylation signal AAUAAA (4,099 nt); the second sequence, AUUUUAAU (3,964 nt) is located 127 nt upstream of a polyadenylation signal and the third CPE, a dodecauridine sequence (2,988 nt), is located 1.1 kb upstream of a polyadenylation signal. Two other polyadenylation signals (boxed in Fig. 1A) were identified at 1,637 nt and 782 nt, respectively.

SSEC P homologs

To determine whether the SSEC P sequence was homologous to any known genes, the polypeptide sequence deduced from SSEC P cDNA was compared with those listed in the GenBank/EMBL database. Two homologous mouse sequences, pYMT2/B (Bishop and Hatat, 1987) and PC11 (Nishioka, 1988), each representative of a gene distributed in more than 150 copies on the long-arm of the Y chromosome, were identified. Expression of this multicopy gene, recently renamed Y-linked spermiogenesis-specific transcript (*Ssty*), is limited to the testis, with expression confined mostly to the round spermatid (Bishop, 1992; Burgoyne et al., 1992; Conway et al., 1994). There is 53% identity between SSEC P and this homolog throughout the entire open reading frame (Fig. 1B). Among the potential phosphorylation sites detected, a protein kinase C phosphorylation site (Ser 204) and a tyrosine phosphorylation site (Tyr 232) are conserved between SSEC P and *Ssty*. An RNA-binding sequence, RNP-1 (AA 218-227) is also conserved (Burd and Dreyfuss, 1994).

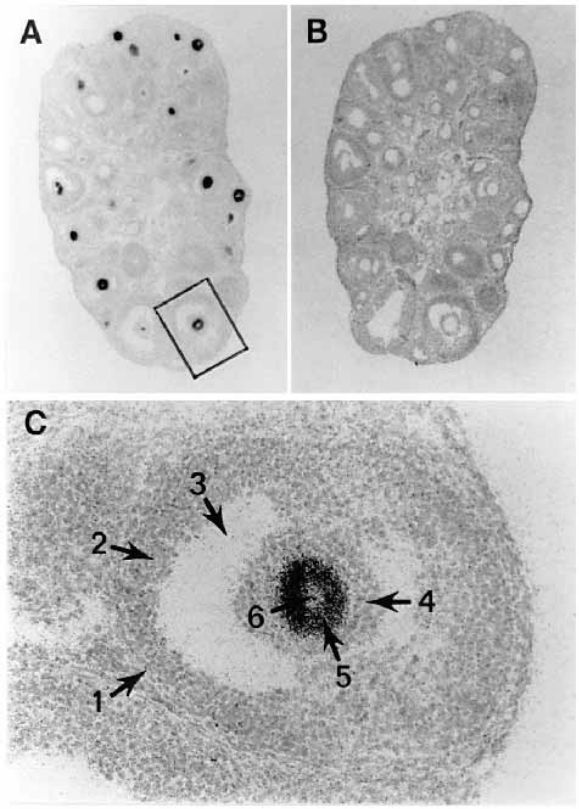

Fig. 2. In situ hybridization of a mouse ovary section. An ovarian section from a 3-week-old mouse was probed with a radiolabeled antisense RNA corresponding to the SSEC P open reading frame (*Bam*HI-*Hin*dIII, 128 nt-840 nt, see Fig. 3B for the restriction map of SSEC P cDNA) and lightly counterstained with hematoxylin. (A) SSEC P antisense probe (50×); (B) SSEC P sense probe (50×); (C) higher magnification of the boxed region of (A) (200×). 1, follicular theca cell layer; 2, granulosa cells; 3, antral cavity; 4, cumulus cells; 5, oocyte; 6, nucleus of oocyte.

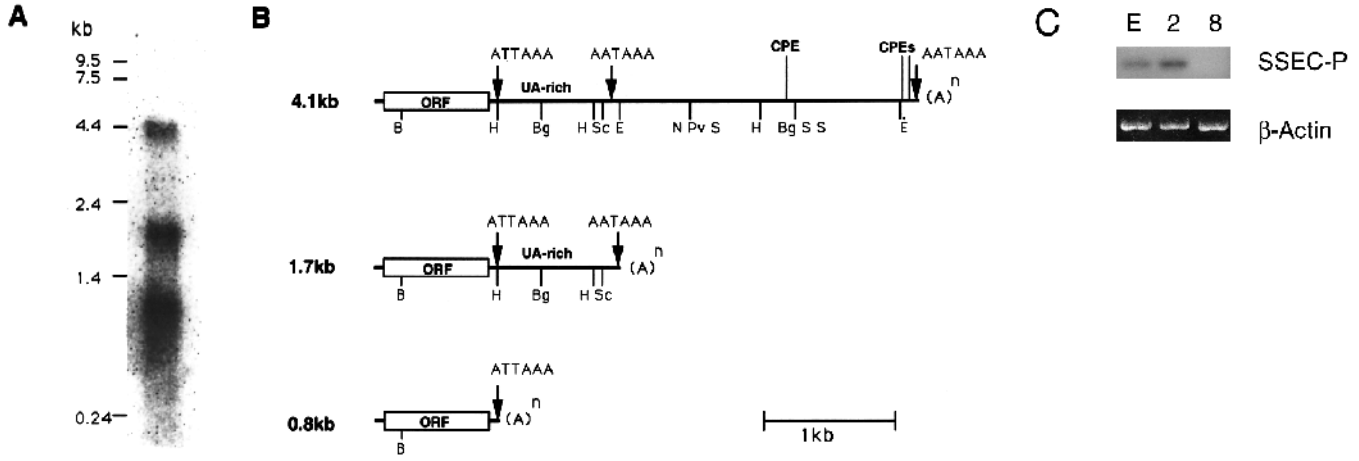

Fig. 3. Analysis of SSEC P transcripts expressed in the full-grown oocyte. (A) A northern blot of full-grown oocytes hybridized with a SSEC P open reading frame probe (*Bam*HI-*Hin*dIII, 128 nt-840 nt). Molecular weight is estimated from an RNA molecular weight marker (GibcoBRL). (B) cDNA structures of three SSEC P transcripts expressed in oocytes. The three polyadenylation signals are marked by arrows, and the three cytoplasmic polyadenylation elements (CPEs) are indicated by vertical lines (for detailed location refer to Fig. 1). UA-rich, indicates CPE-like UA-rich sequences scattered in the 0.9 kb (748 nt-1660 nt) of the 3′UTR region; ORF, open reading frame. Restriction enzymes; B, *Bam*HI; H, *Hin*dIII; Bg, *Bgl*II; Sc, *Sca*I; E, *Eco*RI; N, *Nco*I; Pv, *Pvu*II; S, *Sph*I. (C) RT-PCR from unfertilized egg (E), 2- and 8-cell-stage embryo. SSEC-P (445 bp) was detected by autoradiography and β-actin (539 bp) by ethidium bromide.

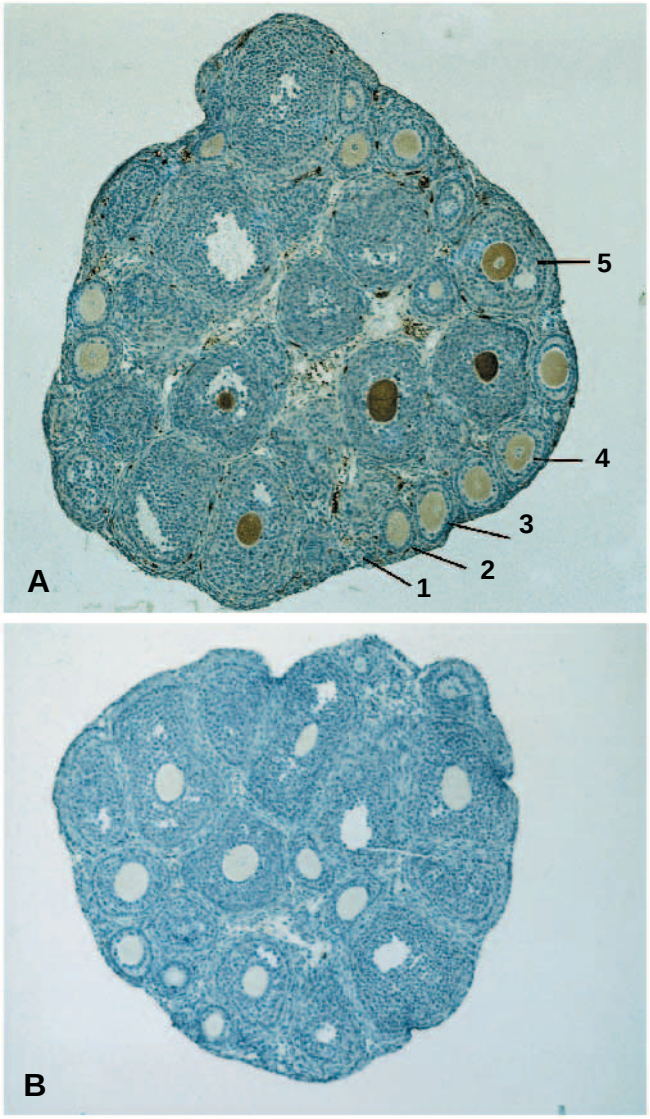

Fig. 4. Immunolocalization of the SSEC P protein in the mouse ovary. An ovarian section from a 3-week-old female mouse was incubated with SSEC P antibody, the reaction was visualized with peroxidase and DAB, and the slide was lightly counterstained with hematoxylin. (A) SSEC P rabbit immune serum (50×); 1, primordial follicle; 2, granulosa-single layer follicle; 3, double layer follicle; 4, multiple layer follicle; 5, antral follicle. (B) rabbit preimmune serum (50×).

A human genomic DNA sequence localized on the X-chromosome, Xp21.1-Xp11.3 (unpublished, Genbank X66465), shows 76% identity at the nucleotide level to the coding sequence of SSEC P (326-709 nt). We also found several human homologous cDNA clones in the WashU-Merck EST and TIGR database. Interestingly, some of sequences from those human clones exhibit more than 80% identity with the 3′UTR of SSEC P. The two phosphorylation sites at Ser 204 and Tyr 232 and the RNP-1 sequence are also conserved in the human homolog. Hybridization analysis of zoo blots (data not shown) suggests that SSEC P is more widely conserved among vertebrates.

Germ cell expression

To determine whether SSEC P is expressed in the female germ cell, mouse ovary sections from 3-week-old mice which contain oocytes at all stages of their growth cycle were hybridized with a SSEC P antisense riboprobe (Fig. 2). Follicular oocytes were specifically labeled, indicating that SSEC P is actively transcribed in these developing germ cells. In fact, SSEC P was expressed in all stages of growing oocytes but not in the primordial oocyte, suggesting that SSEC P gene transcription initiates with oocyte growth. No specific hybridization of the SSEC P probe was found in testicular sections (data not shown).

Northern blot analysis of full-grown oocytes revealed three different SSEC P transcripts of 4.1 kb, 1.7 kb and 0.8 kb (Fig. 3A). Each transcript was further characterized by isolating and sequencing the corresponding cDNAs from the 2-cell-stage cDNA library (Fig. 3B). These transcripts have identical open reading frames but different lengths of the 3′UTR. It appears that each transcript is produced using a different polyadenylation site (boxed sequences in Fig. 1A). The 4.1 kb transcript contains all three of the identified CPE sequences in its 3.3 kb 3′UTR, the 1.7 kb transcript has several CPE-like UA-rich sequences in its 0.9 kb 3′UTR, while the 0.8 kb transcript has no known CPE or UA-rich sequence in its 54 base 3′UTR. These results suggest that SSEC P expression may be post-transcriptionally regulated.

To determine whether SSEC P is expressed in a stage-specific fashion in vivo, RT-PCR was performed on RNA isolated from egg, 2-cell and 8-cell-stage embryos. SSEC P was found to be expressed in the egg and 2-cell but not 8-cell-stage embryo (Fig. 3C).

Intracellular localization of SSEC P-encoded protein

To study the subcellular localization of the SSEC P-encoded protein, polyclonal antibody was raised in a rabbit injected with bacterially expressed SSEC P. Mouse ovarian sections were immunostained with SSEC P antibody using a peroxidase-conjugated secondary antibody and the chromosomes were counterstained with hematoxylin. This protein specifically localized in the oocyte, accumulated in the cytoplasm as the oocyte develops within the ovarian follicle, and was present in highest amounts in the antral and atresic oocytes (Fig. 4). Strikingly, when the oocyte resumes the meiotic cell cycle, some of the SSEC P antibody localized to the meiotic spindle where it was detectable in prometaphase (Fig. 5B), and became more prominent in metaphase, anaphase and telophase (Fig. 5C-E). In the polar body, most of the SSEC P antibody bound the spindle during telophase (Fig. 5E); however antibody reactivity became detectable in the polar body cytoplasm in the metaphase II oocyte (Fig. 5F). Because of the spindle association of a portion of this protein, we designated this gene spindlin (*Spin*). Confocal microscopy revealed reactivity with SSEC P antibody in spindle microtubules and poles of the ovulated oocytes (Fig. 6).

Post-translational modification of spindlin during meiotic and mitotic cell cycles

To determine whether post-translational modification might explain the differences in subcellular localization of spindlin during the meiotic cell cycle, full-grown oocytes and ovulated oocytes were [^{35}S]methionine-labeled and immunoprecipitated with SSEC P antibody. A single SSEC P polypeptide of approx-

imately 28×10^3 M_r was detected in full-grown oocytes arrested in meiotic prophase I (Fig. 7A). In contrast, three bands were observed in metaphase II-arrested ovulated oocytes, indicating that spindlin is post-translationally modified during oocyte development. These immunoprecipitated bands comigrated with three major polypeptides (marked by bars) that are specifically removed after immunodepletion of the extracts with SSEC P antibody (Fig. 7A). To determine whether modification of spindlin is a consequence of phosphorylation, oocytes from both stages were labeled with [^{32}P]orthophosphate and immunoprecipitated with SSEC P antibody (Fig. 7B). A radiolabeled band(s) was discernible in the ovulated oocyte sample, but not in the full-grown oocytes, suggesting that phosphorylation of spindlin marks oocyte meiotic maturation.

To examine the modification of the spindlin protein during the first mitotic division, zygotes were collected from superovulated females caged with males for only 1 hour. These fertilization-synchronized zygotes were [^{35}S]methionine-labeled for 2 hours and immunoprecipitated with SSEC P antibody (Fig. 8). Two of the three previously described polypeptide complexes, i.e., those migrating at approximately 30×10^3 M_r and 35×10^3 M_r, showed a cell-cycle-dependent change in gel mobility (Fig. 8A; Howlett, 1986). Spindlin polypeptides, immunoprecipitated with SSEC P antibody from ovulated oocytes, comigrated with those in the 30×10^3 M_r complex (Fig. 8A, first lane IP). Spindlin showed a faster gel mobility after fertilization (compare 5 and 11 hours in Fig. 8B), concomitant with a similar change in the 30×10^3 M_r complex. During metaphase, migration of spindlin appeared to become slower (Fig. 8C, 16 and 18 hours; Howlett, 1986) obtained similar results after synchronizing zygotes by in vitro fertilization. Our results from eggs fertilized in vivo recapitulate those of Howlett. However, the shift from the 28×10^3 M_r to 30×10^3 M_r form is not complete, perhaps due to the slight variation in the time of fertilization of each egg and the difference between in vivo and in vitro development of the embryos. These observations suggest that post-translational modification, most likely by phosphorylation, may be related to the association of spindlin with the spindle.

To confirm that spindlin represents the polypeptides of the 30×10^3 M_r complex, immunoprecipitates from ovulated oocytes were analyzed by two-dimensional gel electrophoresis (Fig. 9). The pattern of spindlin spots in the two-dimensional gel is the same as that reported for the 30×10^3 M_r complex (Howlett, 1986). In addition, zygotes were cultured in nocodazol to arrest them at metaphase, and spindlin was analyzed by two-dimensional gel electrophoresis after labeling with [^{35}S]methionine. The pattern of spindlin spots in the zygote undergoing at the time of the first mitotic metaphase is the same as that detected at meiotic metaphase (Fig. 9A, data not shown). Based on these results, we postulate that *Spin* encodes the previously described

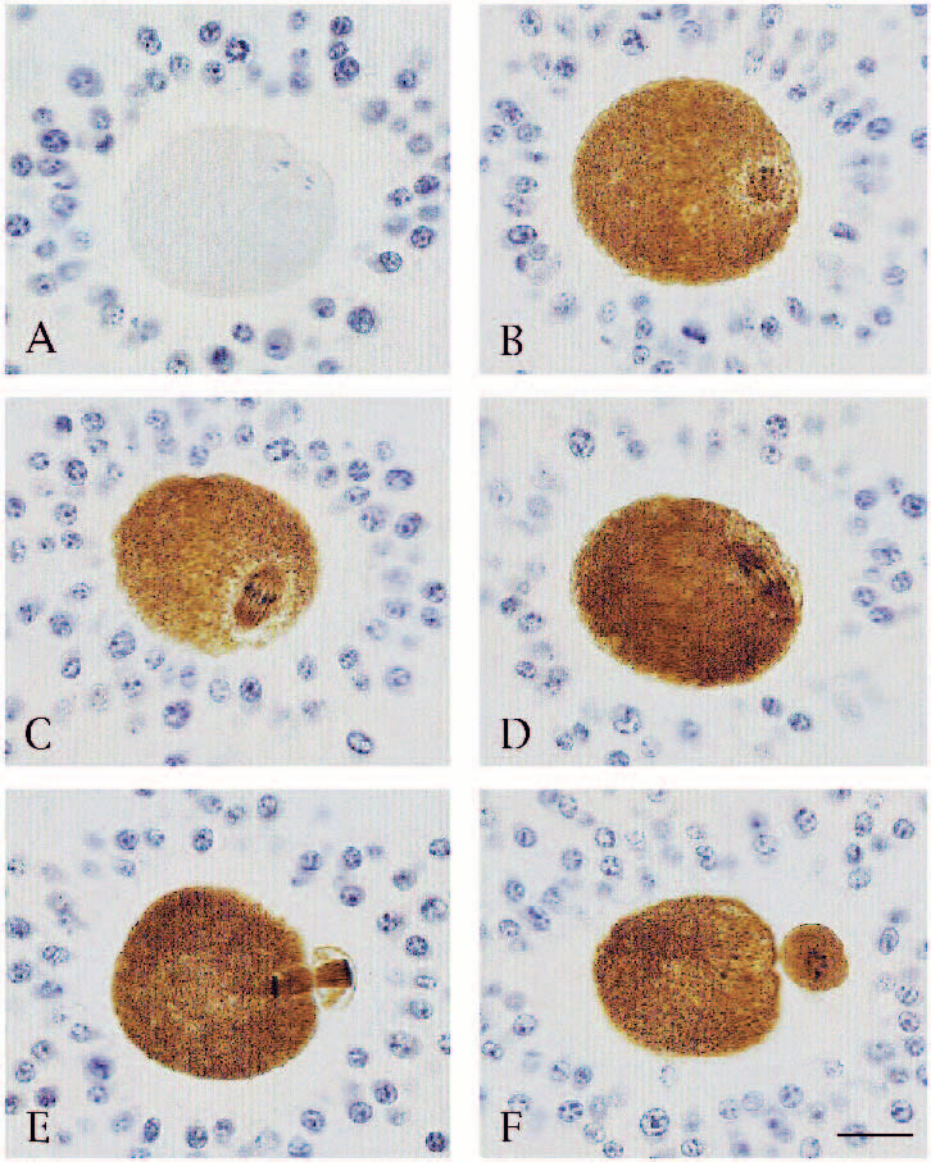

Fig. 5. Immunohistochemical localization of SSEC P to the cytoplasm and spindle of oocytes undergoing meiotic division. Mice were injected with PMSG, followed 48 hours later by injection of hCG and sacrificed after 10 hours. Ovarian sections were incubated with prebleed normal rabbit serum (A) or rabbit SSEC P antiserum (B-F), and antibody binding was visualized by the peroxidase reaction. Slides were counter-stained with hematoxylin to visualize the chromosome of the oocyte and the nucleus of the surrounding follicular cell. A, anaphase oocyte (control); B, prometaphase I; C, metaphase I; D, anaphase I; E, telophase I; F, metaphase II. Bar is 25 μm.

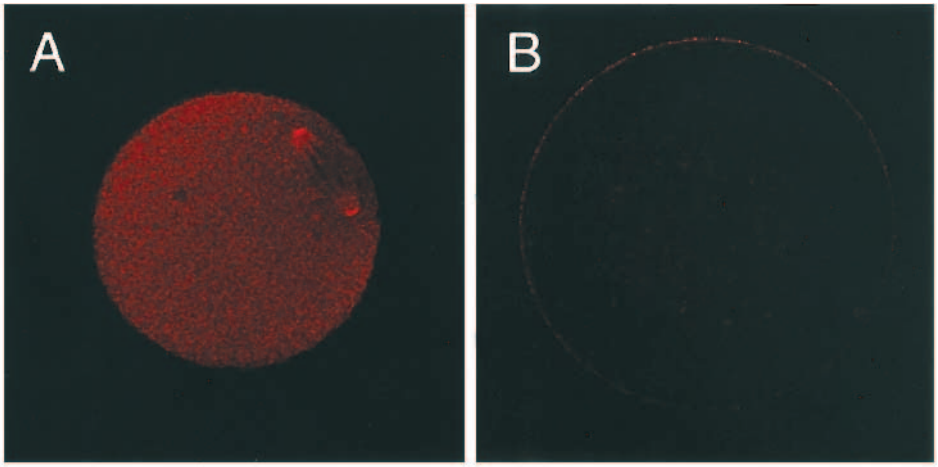

Fig. 6. Localization of the SSEC P product in ovulated oocytes by whole-mount staining and confocal microscopy. Ovulated oocytes were fixed and permeabilized before incubation with SSEC P antibody (A) or prebleed serum (B). Goat anti-rabbit serum conjugated with Cy3 fluorescent dye was used for visualization.

30×10^3 M_r complex phosphorylated in metaphase during the first mitosis.

DISCUSSION

Spin gene family

Few genes expressed during the transition between the gamete and the pluripotent cell of the mammalian preimplantation embryo have been isolated or functionally characterized. The *Spin* gene bears strong homology to the testis-specific gene, *Ssty*, throughout the entire open reading frame, suggesting that *Spin* and *Ssty* may be members of a gene family. Spindlin transcripts are very abundant (0.35% of the total mRNA) and spindlin itself is one of the most abundant proteins biosynthesized in the egg and zygote (Figs 8, 9). Interestingly, more than 150 copies of *Ssty* are scattered over the Y-chromosome long arm (Bishop and Hatat, 1987; Nishioka, 1988). Moreover, when *Ssty* expression is reduced in sex-reversed mice produced by translocation of a partial Y-chromosome, the sperm heads are abnormal, leading to the notion that *Ssty* gene amplification may be required for normal gametogenesis. (Burgoyne et al., 1992). These findings suggest that the gene products of the *Spin* gene family function in a dosage-dependent manner.

Two phosphorylation sites are conserved within the *Spin* gene family and spindlin is phosphorylated during oocyte meiotic maturation (Fig. 7). One subunit of the two consensus RNA-binding motif, RNP-1, is also conserved in these members of the *Spin* family. RNA-binding proteins are known to regulate timely translation and localization of maternal transcripts in other species (Hake and Richter, 1994; Lantz et al.,

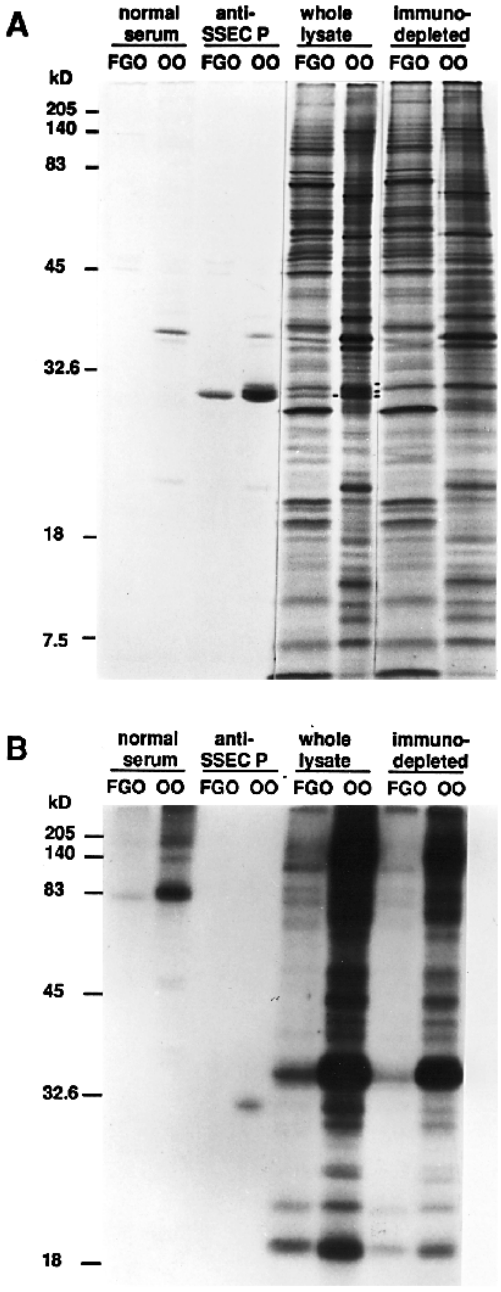

Fig. 7. Modification of the SSEC P protein during oocyte meiotic maturation. (A) Immunoprecipitation of [^{35}S]methionine-labeled germinal vesicle-intact full-grown oocytes (FGO) and metaphase II-arrested ovulated oocytes (OO) with anti-spindlin (SSEC P) antibody using protein A-Sepharose beads. Normal serum, rabbit serum; anti-SSEC P, spindlin antibody; whole lysate, untreated; immunodepleted, cell lysate remaining after immunoprecipitation twice with spindlin antibody. Bars in whole lysate lanes indicate bands that disappeared in immunodepleted samples.
(B) Immunoprecipitation of [^{32}P]inorganic phosphate-labeled ovulated oocytes with spindlin antibody.

1994); however, expression of RNA-binding proteins has not been previously reported in the mammalian embryo.

Spin and *Ssty* are expressed in the maturing gametes in ovary and testis, respectively. Many genes expressed in mammalian testes are also expressed in ovarian oocytes, suggesting that both male and female germ cells may share essential molecules for normal gametogenesis. However, we know of no other case in which two different genes with similar peptide sequences are differentially expressed in the testis and ovary. In addition, although the spindlin transcript and protein are already detected from the time oocyte growth initiates until the 2-cell stage, *Ssty* message is found mainly in the round spermatid, a postmeiotic male germ cell. Like *Spin*, *Siah*-2, a vertebrate homolog of the Drosophila *sina* gene (Della et al., 1995), and cyclin B1 (Chapman and Wolgemuth, 1992) are detected in growing oocytes. However, like *Ssty*, both *Siah*-2 and cyclin B1 are mainly expressed in the round spermatids. It is possible that each gene has different functions in the male and female germ cell (Chapman and Wolgemuth, 1992). The tightly controlled expression pattern of these genes, together with the fact that spindlin transcripts dwindle in the late 2-cell embryo and are not immediate products of embryonic genome activation, suggests that they play a distinct role in the gamete and during the transition from gamete to embryo.

Implications of *Spin* cDNA structure for translational regulation

Multiple transcripts of a single gene, resulting from alternative splicing of the primary transcript or from differential promoter or polyadenylation signal usage, provide one way to modulate

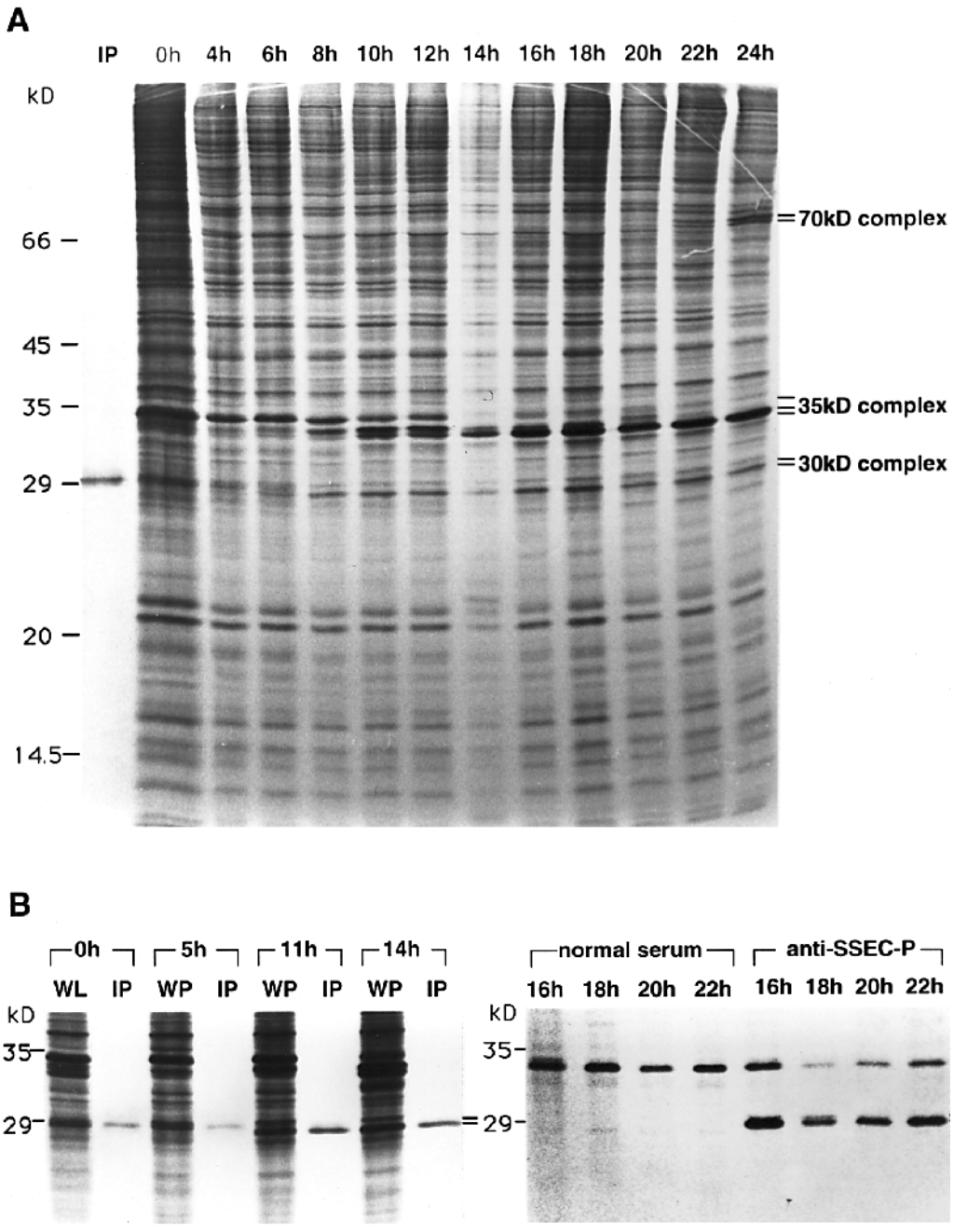

Fig. 8. Modification of spindlin during the first mitotic cell cycle after fertilization. (A) Whole lysates of embryos killed at the indicated times after fertilization, except lane 1, which is an immunoprecipitate of unfertilized eggs with anti-spindlin (SSEC P) antibody (the same sample as that in the 0 hour whole lysate lane). The 30×10^3 M_r and 35×10^3 M_r complex represent polypeptides modified in a cell-cycle-dependent manner during the first mitotic division (Howlett, 1986). The 70×10^3 M_r complex is a set of polypeptides newly synthesized in the 2-cell embryo (Poueymirou and Schultz, 1987).
(B,C) Immunoprecipitation of spindlin during the first mitotic cell cycle. WL, whole lysate; IP, immunoprecipitate with anti-spindlin antibody. Times are hours postfertilization.

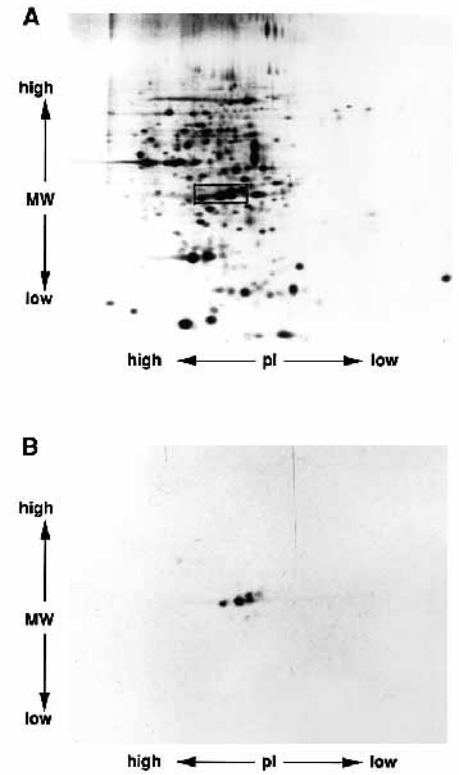

Fig. 9. Two-dimensional protein gel electrophoresis of spindlin. (A) Ovulated oocytes were labeled with [^{35}S]methionine and the lysate was separated by a isoelectric focusing gel for the first dimension and by SDS-polyacrylamide gel for the second dimension. A box is drawn around spindlin. (B) Spindlin after immunoprecipitation of ovulated oocyte lysate separated by two-dimensional gel electrophoresis.

expression of a single gene. In the case of alternative polyadenylation signal usage, different 3′UTR sequences accompany the same open reading frame, perhaps enabling translation of the gene in a changing cellular environment (Leff and Rosenfeld, 1986; Al Ubaidi, 1990; Saez et al., 1990; Code and Olmstead, 1992). This feature is especially important when gene expression is inhibited at the transcriptional level as it is in the egg and zygote. There are three *Spin* transcripts produced by differential usage of polyadenylation signals so that a different 3′UTR controls the activation for translation of each. Previous studies in both Xenopus and mice have clearly shown that activation of maternal transcripts through cytoplasmic polyadenylation controls their translational activity (Fox et al., 1989; McGrew et al., 1989 Huarte et al., 1987). β-actin and tubulin are transcribed and translated at the same time in the oocyte, and these transcripts are slowly degraded during oocyte maturation (Paynton et al., 1988). Translation of the 0.8 kb *Spin* transcript may be regulated in a similar manner, ensuring spindlin synthesis in the growing oocytes. On the contrary, tissue type plasminogen activator (tPA) and hypoxanthine-guanine phosphoribosyl transferase (HPRT), containing CPE sequences within their 3′UTR, are transcribed in the growing oocyte but are not translated until oocyte meiotic maturation (Huarte et al., 1987; Paynton et al., 1988). The 4.1 kb and 1.7 kb *Spin* transcript contains CPEs or CPE-like UA-rich sequences, which are known to regulate translational activation during oocyte maturation (Salles et al., 1992; Gebauer et al., 1994). Moreover, the 4.1 kb transcript contains a dodecauridine sequence (2,988 nt), which is involved in embryonic activation of maternal transcripts in *Xenopus* (Simon et al., 1992; Simon and Richter, 1994). Thus, the different *Spin* transcripts may ensure continuous synthesis of spindlin from the time that oocyte growth initiates to the activation of the embryonic genome in the early embryo.

Spindle association, cell-cycle-dependent phosphorylation and the 30×10^3 M_r metaphase complex

A key finding from this investigation is that spindlin is phosphorylated in a cell-cycle-dependent fashion and associates with the spindle. Phosphorylation and dephosphorylation cascades control the progression of the cell cycle. p34^{cdc2}, the catalytic unit of MPF, phosphorylates nuclear lamin and histone H1 molecules, leading to the dissolution of the nuclear membrane and chromosome condensation, respectively (Langan et al., 1989; Peter et al., 1990). The serine/threonine kinase p39^{mos} appears to be involved in the G_2/M transition through its indirect interaction with the mitogen-activated protein (MAP) kinase (Haccard et al., 1993; Verlhac et al., 1996). It also phosphorylates tubulin, the microtubular subunit that forms the spindle during metaphase (Zhou et al., 1991; Yew et al., 1992) and stabilizes cyclin by direct phosphorylation (Roy et al., 1990). Interestingly, these regulatory kinases associate with the microtubules. For example, p39^{mos} and p34^{cdc2} associate with tubulin (Zhou et al., 1991; Verde et al., 1990; Zhou et al., 1992); the regulatory component of MPF, *cyclin* B, is associated with the spindle (Pines and Hunter, 1991; Bailly et al., 1992; Ookata et al., 1993) and MAP kinase associates with the spindle poles (Verlhac et al., 1993). Apparently, the spindle is the vehicle for the regulatory kinases during metaphase and it is possible that spindlin is the substrate of one of them.

We demonstrated that spindlin is the 30×10^3 M_r metaphase complex detected in the mouse egg and zygote (Howlett, 1986). Since this protein complex is so abundantly synthesized and migrates differentially in a cell-cycle-dependent manner during early embryo development, it has been repeatedly detected in numerous studies of oocyte and preimplantation embryo over the last two decades (Levinson et al., 1978; Cullen et al., 1980; Van Blerkom, 1981; Magnuson and Epstein, 1981; Bolton et al., 1984; Howlett and Bolton, 1985; Rime et al., 1989; De Pennart et al., 1993; Chesnel et al., 1994). Biosynthesis of the 30×10^3 M_r protein gradually decreases after the 2-cell stage and is not detected in the 8- to 16-cell-stage embryo, suggesting that this protein is actively synthesized from maternal transcripts but not from the zygotic genome (Cullen et al., 1980; Bolton et al., 1984). The 30×10^3 M_r protein was suggested to be modified by phosphorylation and perhaps by glycosylation (Magnuson and Epstein, 1981; Van Blerkom, 1981; Howlett, 1986). A requirement for this protein in the initiation and progression of oocyte meiotic maturation has been suggested based on the tight coupling of spindlin synthesis and modification in the oocyte (Rime et al., 1989; De Pennart et al., 1993; Chesnel et al., 1994). The availability of an antibody reagent to the 30×10^3 M_r protein, spindlin, will enable analyses of the

function of the protein during this unique time in the development of the organism.

In summary, we have identified and characterized a gene, *Spin*, encoding the 30×10^3 M_r protein in the oocytes and zygotes. *Spin* defines a gene family and encodes a most abundant mammalian maternal transcript, which is actively translated during the transition from oocyte to pluripotent early embryo. The spindle association and cell-cycle-dependent phosphorylation of this protein supports the notion that spindlin is involved in the progression of the meiotic and first mitotic cell cycles.

We thank Dr Susan Ackerman, The Jackson Laboratory, for her criticism and support of this investigation. We are also grateful to Dr John Eppig and his colleagues at The Jackson Laboratory for their technical support and scientific suggestions and to Dr Mami Ohsugi, The Max-Planck Institute for Immunobiology, Freiburg, Germany for her help in immunolocalizing the spindlin gene product in ovulated eggs. We would like to acknowledge the enthusiastic participation of Eden Haverfield and Julia Po, summer students at The Jackson Laboratory Program for High School Students, in this study. This work was supported by grants from the NIH/NCI (CA 37225, CA 34196), the ACS (IM 707), the Lalor Foundation (BO) and The Max-Planck Society.

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(Accepted 7 October 1996)

GenBank accession number

The GenBank accession number for the *Spin* sequence reported in this paper is U48972.