

Expression and Activity of p40^{MO15}, the Catalytic Subunit of cdk-activating Kinase, During *Xenopus* Oogenesis and Embryogenesis

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Threonine 161 phosphorylation of p34^{cdc2} and its equivalent threonine 160 in p33^{cdk2} by cdk-activating kinase (CAK) is essential for the activation of these cyclin-dependent kinases. We have studied the expression and associated kinase activity of p40^{MO15}, the catalytic subunit of CAK, during *Xenopus* oogenesis, meiotic maturation, and early development to understand in more detail how cdk kinases are regulated during these events. We find that p40^{MO15} is a stable protein with a half-life >16 h that is accumulated during oogenesis. p40^{MO15} protein and its associated CAK activity are localized predominantly to the germinal vesicle; however, a small but significant proportion is found in the cytoplasm. The amount of p40^{MO15} detected in stage VI oocytes remains unchanged through meiotic maturation, fertilization, and early embryogenesis. Significantly, p40^{MO15} was found to be constitutively active during oogenesis, meiotic maturation, and the rapid mitotic cycles of early development. This suggests that regulation of p34^{cdc2} and p33^{cdk2} activity during cell cycle progression does not involve changes in the level or activity of p40^{MO15}/CAK.

INTRODUCTION

Current understanding of cell cycle control emphasizes the key role played by complexes formed between cyclin-dependent kinases (cdk's) and cyclins (Norbury and Nurse, 1992). The first cdk to be identified was p34^{cdc2}, the product of the CDC2 gene in fission yeast, and its homologue CDC28 in budding yeast. In a complex formed with G2/M-phase specific (B-type) cyclins, homologues of p34^{cdc2} are responsible for controlling entry into meiotic and mitotic M-phases in all eukaryotic cells (Moreno *et al.*, 1989; Nurse, 1990). In yeast, p34^{cdc2} complexed with G1- or S-phase specific cyclins is also required for passage through the start control point in G1 and S phase and may be the only cdk required to drive the cell cycle in these cells (Lew and Reed, 1992; Nasmyth, 1993). In higher eukaryotes the picture is more complex with a family of at least six known cdk's, each of which functions at different points in the cell cycle, partnered by one of the cyclin A, B, D, or E family of proteins (reviewed by Pines, 1993; Sherr, 1993). The precise function of each cdk-cyclin complex has yet to be established, but the increasing amount of information for p34^{cdc2} (=p34^{cdk1}) and the closely related kinase

p33^{cdk2} (Elledge and Spottswood, 1991; Paris *et al.*, 1991) has revealed many aspects of their regulation during cell cycle progression.

The activity, localization, and probably substrate specificity of p34^{cdc2} and p33^{cdk2} are regulated at several levels. First, the periodic expression of essential cyclin subunits during the cell cycle ensures that cdk activation only occurs at the appropriate time and place (Reed, 1992; Sherr, 1993). Second, the catalytic activity of the complex is subsequently controlled by cyclin-dependent phosphorylation and dephosphorylation of specific tyrosine and threonine residues (Norbury *et al.*, 1991; Solomon *et al.*, 1992; Draetta, 1993). Third, small inhibitory proteins have recently been discovered that form stable ternary complexes with cdk-cyclin, inhibiting protein kinase activity and negatively regulating progression through G1 (reviewed by Hunter, 1993).

p33^{cdk2} appears to function predominantly during G1 and S phase (Fang and Newport, 1991; Pagano *et al.*, 1993). Consistent with this, p33^{cdk2} is known to associate only with members of the cyclin E, D, and A families during these phases of the cell cycle (Koff *et al.*, 1991; Rosenblatt *et al.*, 1992). Association with cyclin A, for

example, is accompanied by phosphorylation on a threonine at position 160 (T160), resulting in a 100 000-fold increase in the kinase activity of the complex (Gu *et al.*, 1992; Connell-Crowley *et al.*, 1993). p34^{cdc2}, which is required for entry into M-phase, associates with cyclin A but principally with cyclin B (Pines and Hunter, 1989; Gautier *et al.*, 1990). Studies on the p34^{cdc2}-cyclin B kinase (previously known as maturation-, M phase-, or mitosis-promoting factor [MPF]) in fission yeast and vertebrate cells have provided the most complete description of the mechanisms of cdk regulation. Cyclin B accumulates in the cell during G₂, associates with p34^{cdc2}, and allows the cyclin B-dependent phosphorylation of tyrosine (Y15) and threonine (T14 and T161) residues. T14 and Y15 phosphorylation dominantly inhibits p34^{cdc2} kinase function, whereas T161 phosphorylation is essential for p34^{cdc2} kinase activity (Krek and Nigg, 1991; Norbury *et al.*, 1991). The protein kinase weel1 (and also mik1) is responsible for the inhibitory Y15 phosphorylation during interphase (Lundgren *et al.*, 1991; Parker *et al.*, 1992; McGowan and Russell, 1993), whereas the phosphatase cdc25, which is activated at the end of G₂, dephosphorylates Y15 and activates the p34^{cdc2} causing entry into M phase (Gautier *et al.*, 1991; Millar *et al.*, 1991). Cyclin B degradation during anaphase is followed by the dephosphorylation of T161, kinase inactivation, and exit from M phase (Lorca *et al.*, 1992). In budding yeast the role of Y15 phosphorylation during G₂, entry into M-phase (Amon *et al.*, 1992; Sorger and Murray, 1992), and also the requirement for cyclin degradation for exit from mitosis (Surana *et al.*, 1993) is less clear.

Until recently the kinase responsible for the essential activating phosphorylation of p34^{cdc2} T161 remained unknown, although partial purification of an activity known as cdc2-activating kinase (CAK) from *Xenopus* eggs (Solomon *et al.*, 1992) and cultured mammalian cell extracts (Desai *et al.*, 1992) suggested that a single kinase was responsible for phosphorylation of both T161 on p34^{cdc2} and also the corresponding T160 on p33^{cdk2}. Recent work in our laboratory has resulted in collaborations that have established that a kinase known as p40^{MO15} forms the catalytic subunit of CAK (Fesquet *et al.*, 1993; Poon *et al.*, 1993; Solomon *et al.*, 1993). p40^{MO15} is a 40-kDa protein serine/threonine kinase that has 43% amino acid sequence identity with p34^{cdc2} and is encoded by a *Xenopus* oocyte cDNA clone (MO15) that we isolated 4 y ago (Shuttleworth *et al.*, 1990). Together with our collaborators we have recently shown that in *Xenopus* and starfish oocytes and eggs, p40^{MO15} forms the catalytic subunit of CAK and is the kinase responsible for phosphorylating cdc2 on T161 and cdk2 on T160 (Fesquet *et al.*, 1993; Poon *et al.*, 1993; Solomon *et al.*, 1993). Active p40^{MO15} is present in high molecular weight complexes and requires phosphorylation on a threonine (T176) equivalent to T161 in p34^{cdc2}, sug-

gesting that it associates with other proteins and may be regulated in a similar manner to cdk's.

The ovaries of adult *Xenopus laevis* contain oocytes at various stages of oogenesis, which are classified according to Dumont from stage I (previtellogenic, <0.25 mm diameter) to fully differentiated stage VI (1.2 mm diameter) (Dumont, 1972). During oogenesis, oocytes grow and accumulate stockpiles of various molecules, including proteins and mRNAs that are required for formation of a mature egg and for the events of early embryogenesis. Throughout this period of differentiation, which can last for several years, the oocytes remain arrested in diplotene of prophase meiosis I. Fully grown stage VI oocytes contain significant amounts of inactive p34^{cdc2}-cyclin B2 complex (or "pre-MPF") where cdc2 is phosphorylated on both T161, T14, and Y15, together with a 10-fold excess of monomeric p34^{cdc2} (Gautier and Maller, 1991). Progesterone stimulates activation of p34^{cdc2}-cyclin B (or MPF) and several other kinases including c-mos and mitogen-activating protein kinase (Nebrada and Hunt, 1993), resulting in resumption and completion of meiosis I, followed by arrest in metaphase of meiosis II as mature oocytes (or eggs) containing high levels of p34^{cdc2} activity (Smith, 1989). Stage VI oocytes contain very little p33^{cdk2} or cyclin A protein; however, during meiotic maturation p33^{cdk2}, cyclin B1, and cyclin A are accumulated (Kobayashi *et al.*, 1991; Paris *et al.*, 1991). Fertilization of eggs triggers cyclin degradation, inactivation of p34^{cdc2}, and completion of meiosis II, followed by entry into the rapid mitotic cell cycles of the early embryo. These rapid mitotic cycles involve periodic synthesis and destruction of cyclins that drive the activation and inactivation of p33^{cdk2} and p34^{cdc2} and execution of S and M phase (Murray and Kirschner, 1989).

To understand how p40^{MO15} functions to regulate cdk activity during the varied cell cycle events of oogenesis and embryogenesis, we have investigated its expression and activity in *Xenopus* oocytes and embryos. We present here data that shows that p40^{MO15} is a stable protein that accumulates predominantly in the nucleus during oogenesis. The p40^{MO15} persists at a constant level through meiotic maturation, fertilization, and ≥36 h into early development. Furthermore, we can detect no significant changes in MO15 kinase activity during oogenesis, meiotic maturation, or during the rapid mitotic divisions of early embryogenesis. We conclude that p40^{MO15} is constitutively active in *Xenopus* oocytes and embryos and discuss the possible implications of these observations for regulation of p34^{cdc2} and p33^{cdk2} activity in these cells.

MATERIALS AND METHODS

Oocytes and Embryos

Oocytes stages II–VI were obtained from adult female *Xenopus laevis* frogs and maintained in modified Barth's medium (Barth-X) as de-

scribed in Colman (1984). When oocytes were to be matured by incubation in 5 µg/ml progesterone, the frog was primed 2 d before isolation of oocytes by injection with 100 U of follicle-stimulating hormone. Unfertilized eggs and embryos were obtained as described by Gurdon (1977).

Immunocytochemistry

Ten-micrometer sections of unfixed stage VI oocytes mounted in OCT mounting medium (Tissue-Tek, Raymond Lamb, London, United Kingdom) were taken in a cryostat (Bright Instruments, United Kingdom) at -30°C and fixed briefly in ice-cold acetone. Staining of sections was performed (in a humidity chamber) with MO15-specific immune serum 15SSRV (Shuttleworth *et al.*, 1990) or preimmune serum at 1/50 dilution together with nucleoplasmin-specific mouse ascites supernatant PA3L5 (a generous gift provided by Colin Dingwall, CRC/Wellcome Institute, Cambridge) at 1/100 dilution for 1 h. Sections were washed extensively in phosphate-buffered saline (PBS), 1% fetal calf serum, 0.5% Tween-20 then incubated for 40 min with alkaline phosphatase-conjugated goat anti-rabbit IgG at 1/1000 dilution and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG at 1/100 dilution. Sections were washed, stained using Vector Red substrate staining kit (Vector Laboratories, Peterborough, United Kingdom) for 15 min, and washed thoroughly again. Stained sections were mounted in PBS/glycerol mounting medium containing anti-fade compound (Citiflour, London, United Kingdom) and sealed. Slides were viewed (magnification ×32) using a Leitz diaphan microscope (Rockleigh, NJ). Images were photographed using a photoautomat on Kodak Ektachrome 160T film (Rochester, NY).

Metabolic Labeling of Oocytes with ³⁵S-methionine

Oocytes were incubated in 5 µl per oocyte of Barth-X containing 1 mCi/ml ³⁵S-methionine for 4 h at 23°C. After labeling some samples, where indicated in the experiment, were chased in Barth-X containing 1 mM cold methionine for 16 h.

Protein Analysis

Oocytes and embryos were homogenized in ice-cold RIPA buffer (20 mM tris(hydroxymethyl)aminomethane [Tris]-HCl pH 7.5, 100 mM NaCl, 15 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid pH 8, 1% Nonidet P-40 containing a cocktail of protease and phosphatase inhibitors: 1 mM phenylmethylsulphonyl fluoride, 10 µg/ml leupeptin, 10 µM pepstatin, 80 mM β-glycerophosphate, 1 mM NaF, 1 mM Na vanadate). Homogenates were then centrifuged at 14 000 rpm for 2 min at 4°C, and the pellets were discarded.

Immunoprecipitations were performed either in ice-cold RIPA buffer for samples destined for kinase assays or IP buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulphate [SDS] containing the same cocktail of protease and phosphatase inhibitors as listed above) for samples containing ³⁵S-methionine-labeled protein. Immune complexes were formed using MO15-specific immune serum 15SSRV (Shuttleworth *et al.*, 1990) or preimmune serum (at 1/500 dilution for 1 h at 4°C), immobilized on 10 µl protein A sepharose (1 hour at 4°C on a turning wheel), and washed four times in 100 vol of ice-cold RIPA before kinase assays, or for samples containing ³⁵S-methionine labeled protein, four times in ice-cold IP buffer followed by boiling in 20 µl of 2× sample buffer and analysis by 10% SDS-polyacrylamide gel electrophoresis (PAGE).

Immunoblotting

Homogenates of oocytes and embryos were prepared (as described above) and spun at 14 000 rpm in a microcentrifuge at 4°C for 2 min. The supernatant was resuspended in an equal volume of 2× SDS-PAGE sample buffer, boiled, and electrophoresed on 10% SDS-PAGE. Separated proteins were transferred onto nitrocellulose (Hybond-ECL,

Amersham, Arlington Heights, IL) in 20 mM Tris base, 150 mM glycine, 20% methanol, 0.1% SDS at 100 mA for 1 h in a semidry transfer cell (BioRad, Richmond, CA). The membrane was blocked for 2 h using TBST (10 mM Tris-HCl pH 8, 150 mM NaCl, 0.05% Tween 20) containing 4% dried milk. The membrane was incubated with the anti-MO15 antibody 15SSRV at 1/500 dilution in TBST/4% dried milk for 16 h at 4°C with rocking. The membrane was washed three times for 10 min each with TBST/4% dried milk and incubated with horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin (Amersham) at 1/1000 dilution in TBST/2% dried milk at 23°C for 30 min. The membrane was then washed with four changes of TBST for 30 min with gentle agitation and developed using an enhanced chemiluminescence (ECL) kit (Amersham). Membranes were exposed to autoradiographic film (Hyper-MP, Amersham), and the resulting signal was quantitated using scanning densitometry (Ultra-violet Products, Enhanced Analysis System). In each case a dilution series of recombinant GST15 protein was included as an internal standard. All measurements used were within the linear region of the ECL detection method.

Isolation of Total RNA from Oocytes, Unfertilized Eggs, and Embryos

Total cellular RNA was extracted from oocytes and embryos using a Guanidium/hot phenol protocol. Groups of 10 oocytes or embryos were homogenized in 200 µl 4 M guanidium thiocyanate, 42 mM sodium citrate, 0.83% lauryl sarcosine, 0.2 mM β-mercaptoethanol and incubated at 60°C for 15 min. Two hundred microliters of 0.1 M CH₃COONa pH 5.2, 10 mM Tris-HCl pH 7.5, 1 mM EDTA was added, followed by 400 µl of phenol/chloroform. The mixture was vortexed briefly and incubated at 60°C for 10 min with regular vortexing. The aqueous layer was separated by microcentrifugation at 14 000 rpm for 15 min and reextracted with 400 µl of phenol/chloroform followed by a chloroform extraction. RNA was collected by ethanol precipitation.

Ribonuclease Protection Assay

pMO15 plasmid DNA (Shuttleworth *et al.*, 1990) was digested with *Dra* II deleting 787 base pairs (bp) from the 3' end of the MO15 cDNA, and the parent molecule was religated. This construct was linearized with *Sst* I and used to transcribe a 689 nucleotide (nt) ³²P-labeled antisense cRNA probe with T7 RNA polymerase (Pharmacia, Piscataway, NJ) as described previously (Horrell *et al.*, 1987). p6BC (partial *Xenopus* ornithine decarboxylase cloned into pBluescript KS- vector, a generous gift of Dr. H. Osborne, Rennes) was linearized with *Xho* I and used to generate a 454 nt ³²P-labeled antisense ODC cRNA probe using T7 RNA polymerase.

In vitro transcriptions and ribonuclease protection assays were performed as described by Horrell *et al.* (1987). For each sample, 5 × 10⁴ cpm of gel-purified riboprobe was ethanol precipitated with (10 µg) oocyte or embryo RNA at -20°C for 16 h. After RNase digestion products were analyzed by electrophoresis in a 4% polyacrylamide/8M urea gel followed by autoradiography.

Expression and Purification of Recombinant Proteins from Bacteria

Construction of GST-cdk2 and GST-cdk2T160A (kind gifts of Dr. T. Hunt, Imperial Cancer Research Laboratories, South Mimms, United Kingdom) were described by Tsai *et al.* (1991) and Poon *et al.* (1993). The fusion proteins were expressed in *Escherichia coli* strain BL21(DE3) and purified as described by Poon *et al.* (1993).

CAK Assay by Direct Phosphorylation of GST-cdk2

Samples to be assayed for CAK activity were immunoprecipitated using 15SSRV MO15-specific immune serum or preimmune serum (Shuttleworth *et al.*, 1990) as described above. Kinase activity was

measured by incubating the immune complexes in a volume of 25 μ l containing the following: 1 μ g of purified bacterially expressed GST-cdk2 or GST-cdk2T160A, 15 mM Mg(OAc)₂, 30 μ M ATP and 3 μ Ci [γ -³²P] ATP at 23°C for 45 min with mixing. The reaction mix was separated by microcentrifugation at 14 000 rpm for 2 min at 4°C, and 25 μ l of 2 \times SDS-PAGE sample buffer was added to the supernatant. Samples were boiled for 5 minutes and electrophoresed on 10% SDS-PAGE. Dried gels were analyzed by phosphorimage analysis (Molecular Dynamics, Sunnyvale, CA) to quantitate the relative phosphorylation of GST-cdk2 protein and also exposed to X-ray film.

RESULTS

Expression of MO15 Protein During Oogenesis and Early Development

From previous data we knew that adenylated MO15 transcripts are accumulated during early oogenesis (Shuttleworth *et al.*, 1990). To establish whether these transcripts are being translated, oocytes at various stages of oogenesis were labeled for 4 h in ³⁵S-methionine, homogenized, and immunoprecipitated with MO15-specific 15SSRV antibody (Shuttleworth *et al.*, 1990). Figure 1A shows that p40^{MO15} is synthesized by stage II (lane 1), stage IV (lane 2), and stage VI (lane 3) oocytes. The fate of this newly synthesized p40^{MO15} was followed by incubating ³⁵S-methionine-labeled oocytes for 16 h in excess cold methionine. Figure 1B shows that during the 16-h chase period >85% of the newly synthesized p40^{MO15} remained in all stages of oocyte examined (lanes 1–3). Furthermore, in stage VI oocytes the newly synthesized p40^{MO15} became localized almost exclusively in the germinal vesicle (GV) of stage VI oocytes (lane 6) during the 16-h chase period. It is also clear from Figure 1B that the ³⁵S-methionine-labeled p40^{MO15} remained stable through meiotic maturation (lane 4) and for 90 min after prick-activation (lane 5). Together these results indicate that p40^{MO15} is expressed throughout oogenesis as a stable protein with a t_{1/2} > 16 h and that p40^{MO15} is rapidly localized to the GV in stage VI oocytes.

To establish the steady-state levels of p40^{MO15} accumulated during oogenesis total oocyte protein was investigated by immunoblotting. Figure 2A shows the amounts of p40^{MO15} contained in 25 stage II (lane 1), 12.5 stage IV (lane 2), and 2.5 stage VI oocytes (lane 3). By comparison with known amounts of GST-MO15 fusion protein (unpublished observations), one stage II oocyte is estimated to contain ~7.5 ng, one stage IV oocyte ~12 ng, and one stage VI oocyte 75 ng of p40^{MO15}.

We have demonstrated previously that MO15 transcripts accumulated during oogenesis become deadenylated during meiotic maturation. These deadenylated transcripts persist through early embryogenesis until mid-blastula (stage 8) but are only just detectable after gastrulation (stage 12) (Shuttleworth *et al.*, 1990). In general, the adenylation status of an mRNA reflects its translation status, and indeed the deadenylation of

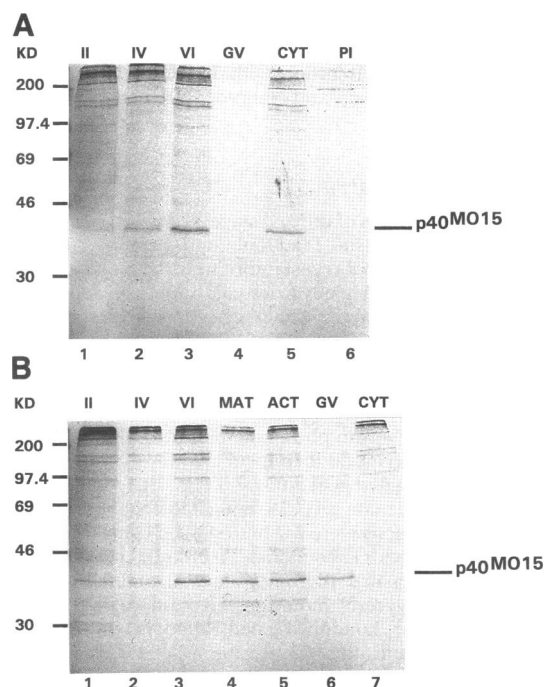


Figure 1. The synthesis and stability of p40^{MO15} during oogenesis and meiosis. (A) Proteins from oocytes labeled for 4 h using ³⁵S-methionine were immunoprecipitated with MO15-specific immune serum and analyzed by SDS-PAGE. The relative amounts of labeled p40^{MO15} in 30 stage II (lane 1), 20 stage IV (lane 2), five stage VI (lane 3), five dissected stage VI oocyte GVs (lane 4), and five dissected stage VI oocyte cytoplasm (lane 5) are shown. Also shown is protein immunoprecipitated from five labeled stage VI oocytes using preimmune serum (lane 6). (B) Oocytes were labeled for 4 h with ³⁵S-methionine then incubated for 16 h in excess cold methionine. In addition, a number of ³⁵S-methionine labeled stage VI oocytes were incubated for 16 h in excess cold methionine in the presence of progesterone. Some of these progesterone-matured stage VI oocytes were then prick-activated and incubated for a further 90 min. Proteins were then immunoprecipitated and analyzed as described in A. The relative amounts of labeled p40^{MO15} in 30 stage II oocytes using preimmune serum (lane 1), 20 stage IV (lane 2), five stage VI (lane 3), five progesterone-matured stage VI oocytes (lane 4), five prick-activated, mature stage VI oocyte GVs (lane 6), and five dissected stage VI oocyte cytoplasm (lane 7) are shown. The positions of molecular weight markers (in kDa) are indicated.

MO15 transcripts is accompanied by a marked reduction of p40^{MO15} synthesis in mature oocytes (Shuttleworth *et al.*, 1990). To investigate the consequences of this reduction in p40^{MO15} synthesis, the amount of p40^{MO15} present in oocytes during meiotic maturation and in embryos during early development was determined by immunoblotting total protein from an equal number of oocytes or embryos. Figure 2B shows that the amount of p40^{MO15} present in mature stage VI oocytes (lane 1) persists at a virtually constant level through fertilization and into early development until at least stage 30 embryos (lanes 2–9). By this stage the embryo has differentiated various tissues, and cells undergo typical somatic cell cycles.

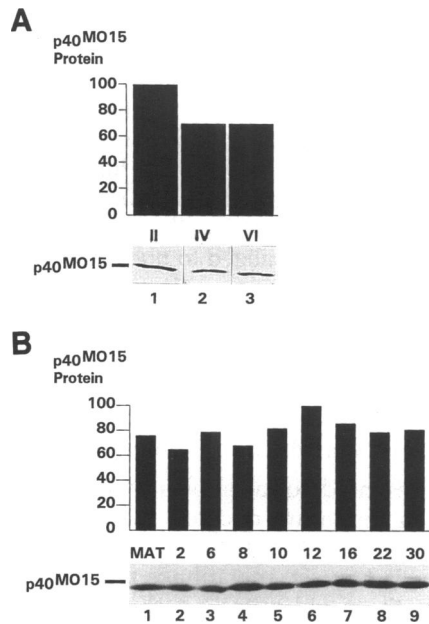


Figure 2. Steady-state levels of p40^{MO15} in oocytes and embryos. (A) Proteins from 25 stage II oocytes (lane 1), 12.5 stage IV oocytes (lane 2), and 2.5 stage VI oocytes were separated by SDS-PAGE and analyzed by Western blotting using MO15-specific immune serum and ECL detection (bottom). The relative amounts of p40^{MO15} present in each sample were quantitated by densitometry and plotted using an arbitrary scale of 1–100. (B) Proteins from five progesterone-matured stage VI oocytes (lane 1), and five stage 2, 6, 8, 10, 12, 16, 22, and 30 embryos (lanes 2–9) were separated by SDS-PAGE and analyzed by Western blotting using MO15-specific immune serum and ECL detection as described in A. The relative amounts of p40^{MO15} present in each sample were quantitated by densitometry and plotted using an arbitrary scale of 1–100.

These results were unexpected in view of the prediction that very little p40^{MO15} would be translated to maintain p40^{MO15} levels during the first 36 h of development (see above), and they suggested that p40^{MO15} is an extremely stable maternal protein with a low rate of turnover. To confirm the previously reported loss in MO15 transcripts during early embryogenesis, we used the more sensitive technique of the RNase protection assay. Figure 3A shows that whereas MO15 cRNA gave rise to a single protected fragment of the predicted size (lane 1), RNA extracted from stage VI oocytes and embryos gave rise to three protected fragments: one at 579 nt, the size predicted for MO15, and shorter fragments at 504 nt and 488 nt. The shorter fragments may be derived from allelic versions of MO15 (*Xenopus* is pseudotetraploid) or from incompletely processed species of mRNA that are known to be present in maternal RNA. Figure 3A shows the amount of MO15 RNA, indicated by the 579 nt and shorter 504 nt and 488 nt fragments in stage VI oocytes (lane 2), unfertilized eggs (lane 3), and stage 8 embryos (lane 4). After stage 8 the level of MO15 RNA drops significantly (7-fold) in stage 10 and 12 embryos (lanes 5 and 6) and is undetectable by this

method from stage 16 to 30 (lanes 7–8). Samples of the same RNA used in Figure 3A were assayed for transcripts of ornithine decarboxylase, a housekeeping gene, and produced a similar signal from each stage of embryogenesis (Figure 3B), indicating that the loss of signal from MO15 transcripts in Figure 3A is not because of degradation of St 16 and St 30 RNA. We conclude from these results that the MO15 transcripts accumulated by stage VI oocytes persist until stage 8 embryos and then drop to undetectable levels by stage 16.

p40^{MO15} Is a Nuclear Protein Kinase

The rapid localization of newly synthesized p40^{MO15} to the GV of stage VI oocytes prompted us to examine the subcellular localization of the p40^{MO15} accumulated by oocytes during oogenesis. Stage VI oocytes were manually dissected, and protein from isolated GVs and cytoplasm was analyzed by immunoblotting. Figure 4 shows that 84% of the p40^{MO15} present in a stage VI

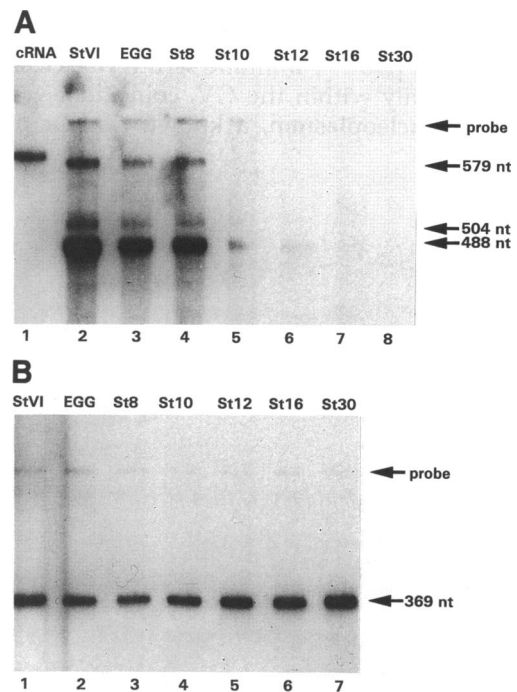


Figure 3. The relative levels of MO15 mRNA in oocytes and embryos. (A) MO15 cRNA (lane 1) and 10 μ g of total RNA extracted from stage VI oocytes (lane 2), unfertilized eggs (lane 3), and stage 8, 10, 12, 16, and 30 embryos (lanes 4–8) were annealed to a 689-nt ³²P-labeled antisense probe from the 5'-end of MO15. After RNase digestion the products were analyzed on a 4% acrylamide/urea denaturing gel. Protected fragments of the predicted size of 579 nt and shorter (504 nt and 488 nt) are indicated. (B) Two micrograms of the total RNA preparations used in A from stage VI oocytes (lane 1), unfertilized eggs (lane 2), and stage 8, 10, 12, and 16 embryos (lanes 3–7) were annealed to a 454-nt ³²P-labeled antisense ornithine decarboxylase RNA probe. After RNase digestion the products were analyzed on a 4% acrylamide/urea denaturing gel. The protected fragment of the predicted size of 369 nt is indicated.

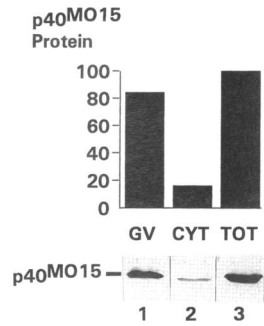


Figure 4. Biochemical localization of $p40^{MO15}$ in stage VI oocytes. Proteins from five dissected stage VI oocyte GV's (lane 1), five dissected stage VI oocyte cytoplasm (lane 2), and five total stage VI oocytes (lane 3) were separated by SDS-PAGE and analyzed by Western blotting using MO15-specific immune serum and ECL detection (bottom). The relative amounts of $p40^{MO15}$ present were quantitated by densitometry and plotted using an arbitrary scale of 1–100.

oocyte is localized to the GV (lane 1), only 16% residing in the cytoplasm (lane 2). Similar values for the distribution of $p40^{MO15}$ were obtained from several experiments (unpublished observations). Although stage VI oocytes can be readily dissected to separate GV's from cytoplasm, it remained possible that $p40^{MO15}$ is a cytoplasmic protein closely associated with the GV. To eliminate this possibility we used indirect immunofluorescence on sections of stage VI oocytes to examine more precisely the localization of $p40^{MO15}$. In Figure 5A the signal from $p40^{MO15}$ immune serum was clearly localized uniformly within the GV, coincident with the signal from nucleoplasm, a known nuclear protein

(Figure 5B). By comparison, staining with preimmune serum gave no signal within the GV (Figure 5C), whereas nucleoplasm was still clearly detected (Figure 5D). Background fluorescence in the cytoplasm was similar using both immune and preimmune serum (Figure 5A, and C). $p40^{MO15}$ therefore has a nuclear and not perinuclear localization. Similarly, using indirect immunofluorescence, we found that the $p40^{MO15}$ present in stage 9 embryos is localized to nuclei, coincident with the distribution of nucleoplasm (unpublished observations). The nuclear localization of $p40^{MO15}$ is therefore not peculiar to oocytes.

$p40^{MO15}$ Is Constitutively Active as a Kinase During Oogenesis, Maturation, and Cleavage

The data presented so far shows that $p40^{MO15}$ protein is present in immature oocytes, mature oocytes, and early embryos. In cell-cycle terms these cells are in prophase meiosis I arrest, metaphase meiosis II arrest, or undergoing the rapid mitotic cycles of early cleavage and clearly might have very different requirements for cdk activation. We have therefore investigated whether $p40^{MO15}$ is active as a kinase in all these situations, using a modification of the assay for CAK described previously (Poon *et al.*, 1993). Bacterially expressed GST-cdk2 does

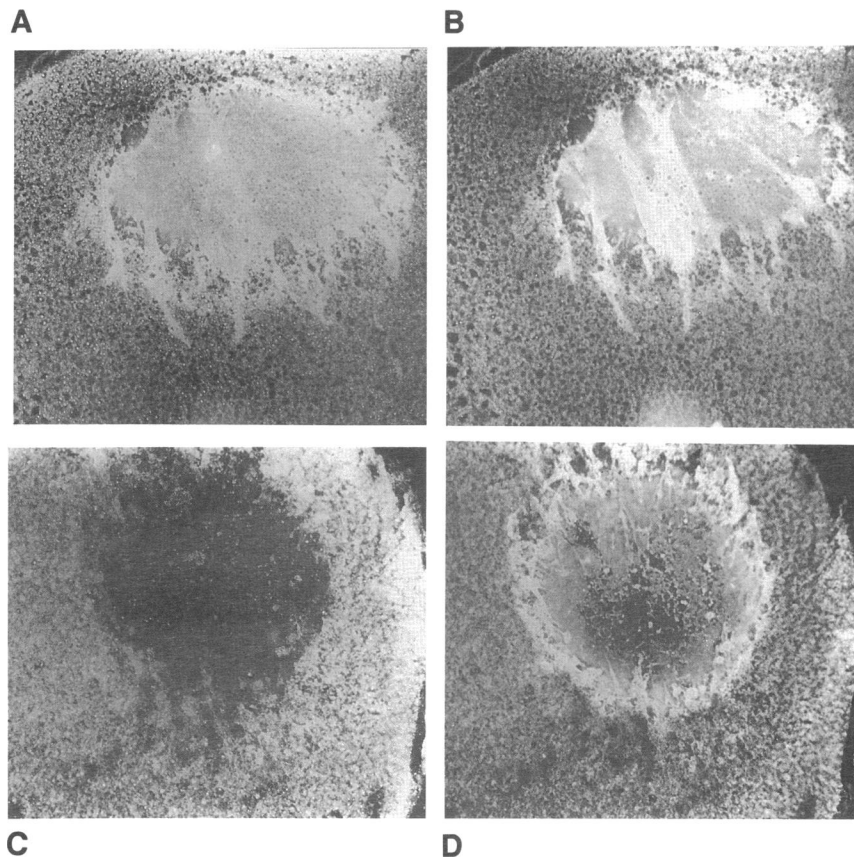


Figure 5. Indirect immunofluorescent localization of $p40^{MO15}$ in stage VI oocytes. Acetone-fixed cryosections of stage VI oocytes were incubated with either MO15-specific rabbit polyclonal antibody together with a mouse monoclonal antibody to nucleoplasm (A and B) or preimmune rabbit serum together with a mouse monoclonal antibody to nucleoplasm (C and D). Rabbit antibodies were detected using alkaline phosphatase conjugated goat anti-rabbit IgG, followed by incubation with Fast Red substrate and fluorescence viewed using a Rhodamine filter (A and C). Mouse antibodies were detected using FITC-conjugated goat anti-mouse IgG and fluorescence viewed using a fluorescein filter (B and D).

not require cyclin A to be phosphorylated on T160 by p40^{MO15}. We therefore assayed immune complexes of p40^{MO15} from oocytes and embryos directly for their ability to phosphorylate purified bacterially expressed GST-cdk2 fusion protein, using mutated nonphosphorylatable GST-cdk2/T160A protein as a control for specificity. Figure 6A shows that p40^{MO15} immune complexes containing equivalent amounts of p40^{MO15} (see Figure 1) from 25 stage II (lane 1), 12.5 stage IV (lane 2), and 2.5 stage VI oocytes (lane 3) showed no significant variation in cdk2 T160 kinase activity. Although on this occasion p40^{MO15} from stage IV oocytes appeared twice as active, this difference is not reproducible and is within the experimental error (because of losses inherent in such a multiple step assay). No significant phosphorylation of GST-cdk2/T160A occurred in the presence of stage VI oocyte immune complexes (lane 4).

Because p40^{MO15} occurs in both the GV (84%) and cytoplasm (16%) of stage VI oocytes (see Figure 4), we next asked whether these two populations of protein were equally active. Oocytes were manually dissected, and immune complexes were prepared from GVs and cytoplasm. Figure 6B shows that 77% of the recovered p40^{MO15} activity was associated with the GV (lane 1) and 23% with the cytoplasm (lane 2), approximating the distribution of p40^{MO15} protein detected by immunoblotting (Figure 4). Again, no significant phosphorylation of GSTcdk2/T160A was detected using immune complexes prepared from total stage VI oocytes (lane 4), whereas immune complexes prepared from stage VI oocytes using preimmune serum gave no significant phosphorylation of GST-cdk2 (lane 5). p40^{MO15} therefore appears to be equally active in both the nucleus and the cytoplasm of stage VI oocytes.

Progesterone stimulation and resumption of meiosis in fully grown oocytes is accompanied by profound changes in p34^{cdc2}-cyclin B activity. Increases in the histone H1 kinase activity of the complex can be detected within 2 h of stimulation. In contrast, we detected no change in the kinase activity of p40^{MO15} after stimulation with progesterone (Figure 6C). p40^{MO15} was equally active in stage VI oocytes before addition of progesterone (lane 1), and 0 (lane 2), 2 (lane 3), and 17 h (lane 4) after addition of progesterone, by which time they had matured and arrested in metaphase of meiosis II.

A similar picture emerges for p40^{MO15} kinase activity during early embryogenesis. p40^{MO15} immune complexes (containing equivalent amounts of p40^{MO15}) prepared from an equal number of cleaving embryos, sampled before mitosis 5 and at ~10-min intervals until mitosis 6 was completed, were assayed for both cdk2 T160 and histone H1 kinase activity. As expected, the levels of histone H1 kinase activity dropped during interphase and increased during M phase (unpublished observations). In contrast, Figure 7A shows no change in the activity of p40^{MO15} during M phase, mitosis 5

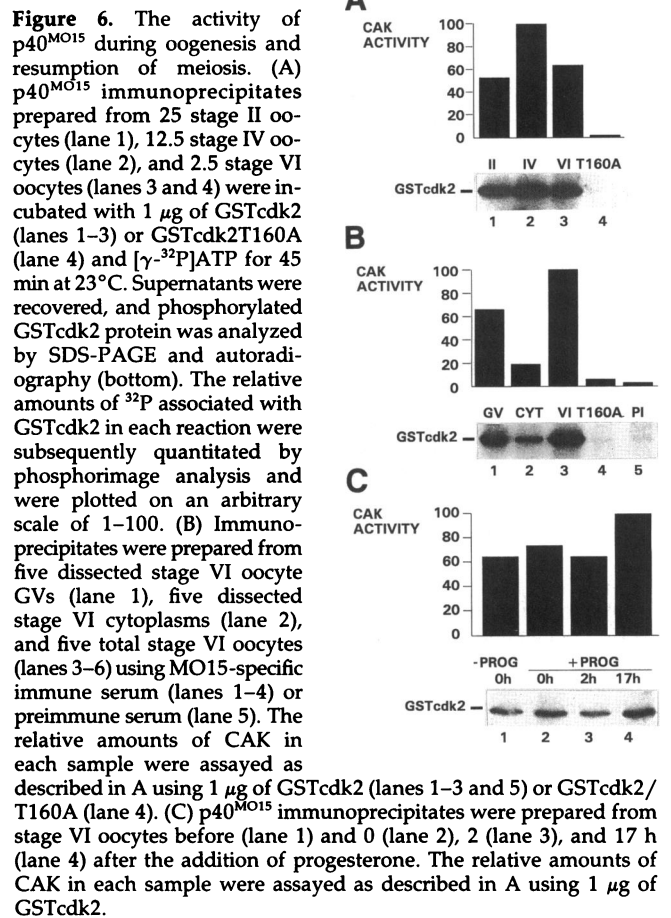


Figure 6. The activity of p40^{MO15} during oogenesis and resumption of meiosis. (A) p40^{MO15} immunoprecipitates prepared from 25 stage II oocytes (lane 1), 12.5 stage IV oocytes (lane 2), and 2.5 stage VI oocytes (lanes 3 and 4) were incubated with 1 μ g of GSTcdk2 (lanes 1–3) or GSTcdk2T160A (lane 4) and [γ -³²P]ATP for 45 min at 23°C. Supernatants were recovered, and phosphorylated GSTcdk2 protein was analyzed by SDS-PAGE and autoradiography (bottom). The relative amounts of ³²P associated with GSTcdk2 in each reaction were subsequently quantitated by phosphorimage analysis and were plotted on an arbitrary scale of 1–100. (B) Immunoprecipitates were prepared from five dissected stage VI oocyte GVs (lane 1), five dissected stage VI cytoplasm (lane 2), and five total stage VI oocytes (lanes 3–6) using MO15-specific immune serum (lanes 1–4) or preimmune serum (lane 5). The relative amounts of CAK in each sample were assayed as described in A using 1 μ g of GSTcdk2 (lanes 1–3 and 5) or GSTcdk2/T160A (lane 4). (C) p40^{MO15} immunoprecipitates were prepared from stage VI oocytes before (lane 1) and 0 (lane 2), 2 (lane 3), and 17 h (lane 4) after the addition of progesterone. The relative amounts of CAK in each sample were assayed as described in A using 1 μ g of GSTcdk2.

(lane 2), interphase (lanes 3–6) to M phase, mitosis 6 (lane 7). The activity of p40^{MO15} is therefore not regulated during the mitotic cell cycle of early cleaving embryos.

Finally Figure 7B shows the CAK activity associated with p40^{MO15} during early embryogenesis. Immune complexes prepared from an equal number of mature stage VI oocytes (lane 1) and stage 2–30 embryos (lanes 2–8) contained equivalent levels of cdk2 T160 activity. The variation between samples (~2- to 3-fold) seen in this experiment was not reproducible and can be attributed to experimental error (losses during the multiple step procedure). p40^{MO15} is therefore equally active throughout early embryogenesis to at least stage 30.

DISCUSSION

To understand more fully the role p40^{MO15} plays in cell cycle control, we have studied the expression of p40^{MO15} protein in *Xenopus* oocytes and embryos. We found that p40^{MO15} is synthesized and accumulated as a stable protein in oocytes throughout oogenesis, consistent with our previous finding that adenylated MO15

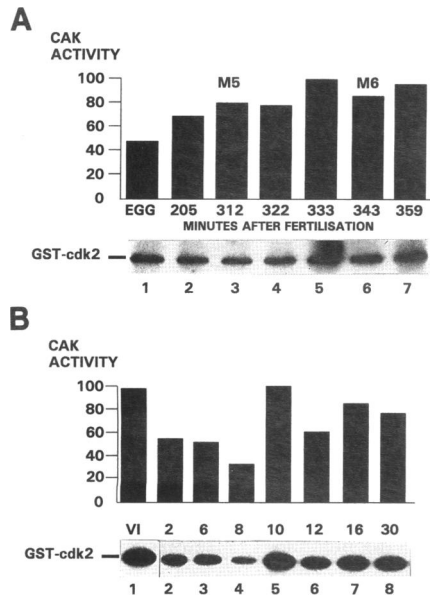


Figure 7. The activity of $p40^{MO15}$ during early embryogenesis. (A) $p40^{MO15}$ immunoprecipitates were prepared from five unfertilized eggs (lane 1) and five cleaving embryos at 205 (lane 2), 312 (lane 3), 322 (lane 4), 333 (lane 5), 343 (lane 6), and 359 min (lane 7) after fertilization and were incubated with $1 \mu\text{g}$ of GSTcdk2 and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 45 min at 23°C . Supernatants were recovered, and phosphorylated GSTcdk2 protein was analyzed by SDS-PAGE and autoradiography (bottom). The relative amounts of ^{32}P associated with GSTcdk2 in each reaction were subsequently quantitated by phosphorimage analysis and plotted on an arbitrary scale of 1–100. Mitosis 5 (M5) occurred after 312 min, and mitosis 6 (M6) occurred after 343 min. (B) $p40^{MO15}$ immunoprecipitates were prepared from five mature stage VI oocytes (lane 1) and five stage 2, 6, 8, 10, 12, 16, and 30 embryos (lanes 2–8), then assayed for CAK activity as described in A.

transcripts are present early in oogenesis (Shuttleworth *et al.*, 1990). The amounts of $p40^{MO15}$ accumulated in stage VI oocytes are maintained at a remarkably constant level through meiotic maturation, fertilization, and early embryogenesis, despite the fact that MO15 transcripts become deadenylated during meiotic maturation (Shuttleworth *et al.*, 1990). They are almost completely degraded after stage 8 (midblastula transition) and undetectable by RNase protection assay by stage 16 onward. MO15 transcripts can be detected by reverse transcription and polymerase chain reaction amplification of RNA from stage 16 to 30 embryos (Shuttleworth, unpublished data) but can only be present at very low levels. Together our data suggests that maternal stockpiles of $p40^{MO15}$ are turned over at a very low rate during the first 36 h of embryogenesis. The stability of $p40^{MO15}$ is not unique to *Xenopus* oocytes and embryos, because ^{35}S -methionine pulse-chase labeling and immunoprecipitation indicate that $p40^{MO15}$ is equally stable (half-life >24 h) in proliferating *Xenopus* WAK cells (Brown, unpublished data). By comparison, the stability of $p34^{cdc2}$ in proliferating mouse 3T3 cells has been reported to vary from a half-

life of 18 h during early G1 to only 7.5 h during G1/S-phase (Welch and Wang, 1992).

It remains possible that a protein closely related to $p40^{MO15}$ is expressed by embryos after the drop in MO15 transcript levels in stage 8 embryos, the point at which zygotic transcription begins. If this were the case, the maternal pools of $p40^{MO15}$ could be replaced by newly synthesized embryonic protein cross-reactive to the anti-MO15 immune serum used. These hypothetical MO15-related transcripts would not necessarily be detected either by our previous Northern blot analysis (using high stringency hybridisation with a 500-bp 5'-end probe) or by RNase protection analysis.

The $p40^{MO15}$ protein present during oogenesis, meiotic maturation, and early embryogenesis appears to be constitutively active. Using a direct assay for the cdk2 T160 kinase activity in $p40^{MO15}$ immunoprecipitates, we have found no significant changes in the relative specific activity of $p40^{MO15}$ present in the nucleus or cytoplasm of prophase-arrested oocytes, maturing oocytes, or rapidly cleaving early embryos. It remains possible that although GST-cdk2 can be phosphorylated by immune complexes of $p40^{MO15}$ in vitro, cyclin association with cdk2 subunits may modulate T160 phosphorylation during cell cycle progression in vivo by altering its affinity or accessibility as a substrate for $p40^{MO15}$. Our in vitro kinase assay, using GST-cdk2 alone as a substrate, may fail to detect changes in the activity of $p40^{MO15}$. However, this seems less likely in view of previous reports that the CAK activity associated with extracts prepared from interphase and M-phase embryos and prophase-arrested oocytes remains constant when assayed using *cdc2*-cyclin B as a substrate (Solomon *et al.*, 1992). Like somatic cells, which accumulate $p34^{cdc2}$ -cyclin B complexes during G2, *Xenopus* oocytes arrested in prophase (at the G2/M boundary) contain a significant pool of cyclin B2 that is complexed with T14-, Y15-, and T161-phosphorylated $p34^{cdc2}$ (Gautier and Maller, 1991). Activation of this $p34^{cdc2}$ -cyclin B2 pool in response to progesterone requires no new synthesis of cyclin B1, B2, or A (Minshull *et al.*, 1991), and dephosphorylation of $p34^{cdc2}$ T14 and Y15 by *cdc25* phosphatase leads rapidly to GV breakdown and completion of meiosis I (Gautier *et al.*, 1991). $p34^{cdc2}$ -cyclin B purified from prophase-arrested starfish oocytes can similarly be activated in vitro by recombinant *cdc25*, confirming that T161 is already phosphorylated (Strausfeld *et al.*, 1991). The existence of an active $p34^{cdc2}$ T161 kinase in prophase-arrested oocytes is therefore entirely consistent with this model. Precise details of the events that lead to inactivation and subsequent reactivation of $p34^{cdc2}$, between meiosis I and meiosis II, have yet to be described. There are obvious differences in the mechanism regulating exit from metaphase during meiosis I compared to mitosis, including the fact that not all cyclins are destroyed after meiosis I (Kobayashi *et al.*, 1991). Therefore it is possible that other processes

unique to meiosis may regulate cdc2 kinase activity. Until the fate of the p34^{cdc2} T161 phosphate is known, we can only speculate that MO15 may be required again after meiosis I to reactivate p34^{cdc2} complexes and enter meiosis II. However, the absolute requirement to maintain the kinase activity of the p34^{cdc2}-cyclin complexes during metaphase meiosis II arrest in mature oocytes/eggs would dictate that p40^{MO15} remains active during this period. Similarly, the requirement to activate p33^{cdk2} and p34^{cdc2} kinase complexes that drive S phase and M phase in the early embryo would again leave very few occasions when the activity of p40^{MO15} is not necessary, assuming that p34^{cdc2} T161 and p33^{cdk2} T160 are dephosphorylated during each cycle. A human homologue of p40^{MO15} has recently been identified and similarly shown to be constitutively active throughout the cell cycle in proliferating human somatic cells (Tassan *et al.*, 1994).

The constitutive activity of p40^{MO15} is in marked contrast to the cell-cycle regulation of wee1 kinase and cdc25 phosphatase activities, even in the rapid mitotic cycles of the frog early embryo that lack many aspects of control present in somatic cell cycles (Kumagai and Dunphy, 1992; Tang *et al.*, 1993). Phosphorylation of p34^{cdc2} Y15, its effect being dominant over T161 phosphorylation, is clearly the preferred mechanism for tightly regulating cdk activity during the transition from G2 into M-phase. The equivalent "fine tuning" of cdk activity at other points in the cycle could be exerted by titration of inhibitory proteins that override T161/T160 phosphorylation and remove the need to tightly regulate CAK activity (Nasmyth and Hunt, 1993).

By direct immunoblotting of dissected oocytes, we found that 84% of p40^{MO15} is localized to the GV of stage VI oocytes. The remote possibility that the small (16%) cytoplasmic population of p40^{MO15} is caused by leakage from the GV during dissection is unlikely but cannot be excluded. Unfortunately, no suitable control exists to confirm the absence of contamination of cytoplasm by soluble GV proteins. Even proteins such as nucleoplasmin, which are exclusively nuclear in somatic cells, are known to be present (~10%) in the cytoplasm of *Xenopus* oocytes. Leakage from the GV is more likely to present a problem after dissection, although this does not appear to compromise the data presented in Figure 4 where virtually all of the p40^{MO15} detected in intact oocytes can be accounted for. Our immunofluorescence studies indicated a uniform distribution throughout the GV. The predicted amino acid sequence near the C-terminus of *Xenopus* p40^{MO15}, and also in human and mouse p40^{MO15} homologues (Erschler *et al.*, 1993; Tassan *et al.*, 1994), contains a putative bipartite nuclear localization signal (Robbins *et al.*, 1991) that may explain this distribution; however, we have not formally tested this possibility. The fact that we find p40^{MO15} in the nuclei of embryos after the midblastula transition (Brown, unpublished data), together with the obser-

vation that p40^{MO15} is localized predominantly to the nucleus during interphase in human somatic cells (Tassan *et al.*, 1994), suggests that it functions as a nuclear kinase in all cell types.

Interpretation of the nuclear localization of p40^{MO15} is problematical and highlights possible gaps in our knowledge of the precise details of events occurring during release from prophase arrest in oocytes and during the G2/M transition in somatic cells. No problem exists for T160 phosphorylation of p33^{cdk2} complexed with either cyclin D, E, or A, all of which are nuclear proteins throughout the cell cycle (Pines and Hunter, 1991; Sherr, 1993). Because somatic cells spend the majority of their time passing through G1, S, and G2 phases of the cell cycle, the nuclear localization of active p40^{MO15} throughout interphase should not be surprising. However, considering p34^{cdc2}-cyclin B, the only other confirmed substrate for p40^{MO15} in *Xenopus*, this discovery is intriguing. Stage VI *Xenopus* oocytes contain significant amounts of p34^{cdc2} and cyclin B2 but very little p33^{cdk2}, cyclin A, or cyclin B1 (Gautier and Maller, 1991; Kobayashi *et al.*, 1991; Paris *et al.*, 1991). All of the cyclin B2 exists in complex with p34^{cdc2} exclusively in the cytoplasm (Gautier and Maller, 1991), consistent with the observation that cyclin B is a cytoplasmic protein until the G2/M transition in human somatic cells (Pines and Hunter, 1991). The presence of a cytoplasmic population of active p40^{MO15} (~20% of the total) in oocytes could ensure that the p34^{cdc2}-cyclin B2 is T161 phosphorylated. Data on the localization of p34^{cdc2} in somatic cells is conflicting, with reports of its distribution only in the nucleus (Riabowol *et al.*, 1989), only in the cytoplasm (Akhurst *et al.*, 1989), in both (Bailly *et al.*, 1989), or with populations able to relocate during the cell cycle (Ookata *et al.*, 1992). The nuclear localization of both p40^{MO15} and cdc25 (Girard *et al.*, 1992) is difficult to reconcile with a simple model for p34^{cdc2}-cyclin B activation, because cyclin B bound to p34^{cdc2} remains cytoplasmic during interphase (Pines and Hunter, 1991) and only moves to the nucleus at the G2/M transition, concomitant with activation of p34^{cdc2}. The presence of relatively small cytoplasmic populations of p40^{MO15} and cdc25 could allow the observed behavior of p34^{cdc2}-cyclin B, triggering the positive feedback loop that has been postulated to initiate rapid activation of p34^{cdc2}. Alternatively, small amounts of p34^{cdc2}-cyclin B may enter the nucleus with the same effect.

We detect a small but potentially significant population of p34^{cdc2} associated with dissected stage VI oocyte GVs (Shuttleworth, unpublished data). This GV population, representing ~10% of the total p34^{cdc2} pool, is indistinguishable from cytoplasmic p34^{cdc2} by its electrophoretic mobility, suggesting that Y15 and T161 phosphorylated forms are present in the GV and are associated with cyclin (Shuttleworth, unpublished data). If this is the case, the identity of the cyclin involved and the function of this GV population of p34^{cdc2} re-

mains open to question. Only cyclin A and the newly identified cyclin B3 localize to the nucleus in interphase cells (Pines and Hunter, 1991; Gallant and Nigg, 1994) and are potential partners for p34^{cdc2}. However, only insignificant (<1 pg) amounts of cyclin A are present in prophase-arrested oocytes (Kobayashi *et al.*, 1991), and the expression of cyclin B3 in these cells has yet to be reported. Provocatively, preliminary data on the kinetics of activation of p34^{cdc2} in progesterone-stimulated *Xenopus* oocytes suggests that H1 kinase activation in the GV precedes activation in the cytoplasm, the proposed location of all p34^{cdc2}-cyclin B2 or pre-MPF (Shuttleworth, unpublished data). Immediately before GV breakdown, the GV-associated histone H1 kinase activity represents ~10% of the total activity present in the oocyte. The precise function and behavior of p34^{cdc2}-cyclin A, particularly during G2 and M-phases, remains unclear. There are clear differences in aspects of its function and behaviour compared to p34^{cdc2}-cyclin B (Lehner and O'Farrell, 1990; Minshull *et al.*, 1990). Notably, p34^{cdc2}-cyclin A, unlike p34^{cdc2}-cyclin B, is a poor substrate for wee1 kinase (Clarke *et al.*, 1992). The lack of Y15 phosphorylation results in rapid activation (presumably in the nucleus by p40^{MO15}) independent of cdc25 activation. The implications of this for the control of G2/M transition and early mitotic events remains to be resolved.

Although MO15 kinase appears to be constitutively active in all situations examined, we cannot rule out the possibility that the *in vivo* activity of p40^{MO15} may not be accurately reflected by the *in vitro* assay of immune complexes. Gel filtration of egg extracts has shown that active p40^{MO15} is found in high molecular weight complexes (Poon *et al.*, 1993). This, together with the observation that recombinant GST-MO15 protein purified from bacteria requires incubation with cell extract to acquire CAK activity (Poon *et al.*, 1993), has led us to propose that p40^{MO15} activity is regulated by association with regulatory subunit(s) and/or phosphorylation of specific residues such as the T176, which is at a position corresponding to p34^{cdc2} T161. Homogenization and immunoprecipitation may alter the activity of p40^{MO15} complexes, if for example regulatory components are lost or gained during the process. Also, in the presence of phosphatase inhibitors we have observed forms of p40^{MO15} with faster mobilities on SDS-PAGE (Brown, unpublished data). We believe these represent extremely labile phosphorylated forms of the kinase. Although we have detected no differences in the activity of p40^{MO15} that can be associated with this phosphorylated form, it is possible that phosphorylation of specific residues, such as a putative p34^{cdc2} phosphorylation site at serine 170, may regulate localization, complex formation, or substrate recognition *in vivo*. We are currently investigating the identity of a 35-kDa protein that specifically coprecipitates with p40^{MO15} from *Xenopus* oocytes and could be a putative regulatory subunit of

CAK. Similarly, two proteins with molecular weights of 32 and 34 kDa have been found to specifically coprecipitate with the human homologue of p40^{MO15} from HeLa cells (Tassan *et al.*, 1994), whereas a 37-kDa protein has been found associated with purified human CAK (Fisher and Morgan, 1994). Studies on the expression, localization, and function of such regulatory proteins, and their association with p40^{MO15} during oogenesis and embryogenesis and during the somatic cell cycle, will be required to understand in more detail how p40^{MO15} function is regulated throughout the meiotic and mitotic cell cycles.

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