Ura+OsmR:Ura-OsmS) was observed. Tight linkage between the *HOG4* and *URA3* genes is consistent with plasmid integration at the *hog4-1* locus.
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27. Tyrosine phosphorylation of proteins was analyzed by immunoblot analysis with immunoglobulin $2b_k$–affinity purified monoclonal antibody (Upstate Biotech, Lake Placid, NY). Cells were grown in liquid SC medium lacking the appropriate supplement (*31*), pelleted, resuspended in YEPD, and incubated for 2 hours before the addition of NaCl or sorbitol in an equal volume of 0.8 M NaCl of 1.4 M sorbitol in YEPD. Preparation of cell extracts, electrophoresis, transfer conditions, and incubation with antibodies were done as described (*26*). For ammonium sulfate precipitations of extracts, cells were incubated for 1 min after the addition of NaCl, collected by centrifugation at 4°C, and lysed by agitation with 1 volume of glass beads and 3 volumes of buffer [20 mM tris (pH 7.4), 20 mM Na_3VO_4, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100, and leupeptin, aprotinin, and pepstatin A (each 10 μg/ml)]. The extract was clarified by centrifugation and mixed with an equal volume of saturated ammonium sulfate. The precipitate formed after 2 hours at 4°C was collected by centrifugation and washed with 50% ammonium sulfate and 1 mM EDTA, and a portion (60 μg of protein) was subjected to immunoblot analysis. The antibody recognized phosphotyrosine on HOG1 protein because phosphotyrosine (1 mM), but not phosphoserine or phosphothreonine, competed for immunoreactivity (*26*). Addition of 0.4 M NaCl to control cultures after boiling in sample buffer had no effect on the immunoreactivity of cell proteins.
28. Mutant strains were in a YPH499 background. Strains JBY10, JBY40, and JBY1041 contain *hog1-Δ1*, *pbs2-Δ1*, and *hog1-Δ1 pbs2-Δ1*, respectively. The pJB17 plasmid was constructed by insertion of *HOG1* into the polylinker of the high-copy 2μ-based pRS424 plasmid (*30*). The pJB12T plasmid was constructed by deletion of a 1.1-kb Bst XI–Bst XI fragment extending from within the 3′-end of *HOG1* to the polylinker of pRS316 and insertion of this truncated allele, *hog1-Δ2*, into the 2μ-based plasmid pRS426 (*30*). The *hog1-Δ2* mutant on a centromere plasmid complemented the OsmS of JBY10.
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PAC-1: A Mitogen-Induced Nuclear Protein Tyrosine Phosphatase

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Tyrosine phosphorylation of proteins is required for signal transduction in cells and for growth regulation. A mitogen-induced gene (*PAC-1*) has been cloned from human T cells and encodes a 32-kilodalton protein that contains a sequence that defines the enzymatic site of known protein phosphotyrosine phosphatases (PTPases). Other than this sequence, PAC-1 is different from several other known related PTPases exemplified by PTP-1b. PAC-1 is similar to a phosphatase induced by mitogens or heat shock in fibroblasts, a yeast gene, and a vaccinia virus–encoded serine-tyrosine phosphatase (VH1). PAC-1 was predominantly expressed in hematopoietic tissues and localized to the nucleus in transfected COS-7 cells and in mitogen-stimulated T cells.

The activation of quiescent peripheral blood T cells by antigens or mitogens stimulates proliferation and the expression of effector functions. Progression through the cell cycle requires the expression of a set of genes that are induced within minutes to hours after binding of the mitogenic agent (*1*, *2*). Immediate early genes have been identified that are transcribed in response to phytohemagglutinin (PHA) and phorbol 12-myristate 13-acetate (PMA) in human peripheral blood T (PBT) cells (*3*, *4*). One such cDNA clone, pAT 120, now referred to as *PAC-1* (phosphatase of activated cells), is transiently expressed during the G1 phase in mitogen-activated PBT cells and in antigen-activated T cell clones (*4*, *5*). The PAC-1 gene is constitutively expressed in T cells infected with the human retrovirus human T cell leukemia virus (HTLV-I), the etiological agent of adult T cell leukemia (*5*).

Structural characterization of two independent, nearly full-length PAC-1 cDNA clones from human PBT cells revealed an encoded polypeptide of 314 amino acids (Fig. 1A) (GenBank accession number L11329). The predicted protein sequence was confirmed by in vitro transcription and translation and by the production of three antibodies to peptides derived from the predicted amino acid sequence that precipitate an endogenous protein of the expected molecular size. A murine cDNA, cloned by cross-hybridization, encodes a very similar protein (Fig. 1A) (GenBank accession number L11330). The PAC-1 protein has 13 cysteines in humans and 14 cysteines in mice, 12 of which are conserved in position between the two species.

A search of GenBank revealed similarity between amino acids 250 and 270 of the PAC-1 protein sequence to several protein tyrosine phosphatases (PTPases) in a region that is the most highly conserved among

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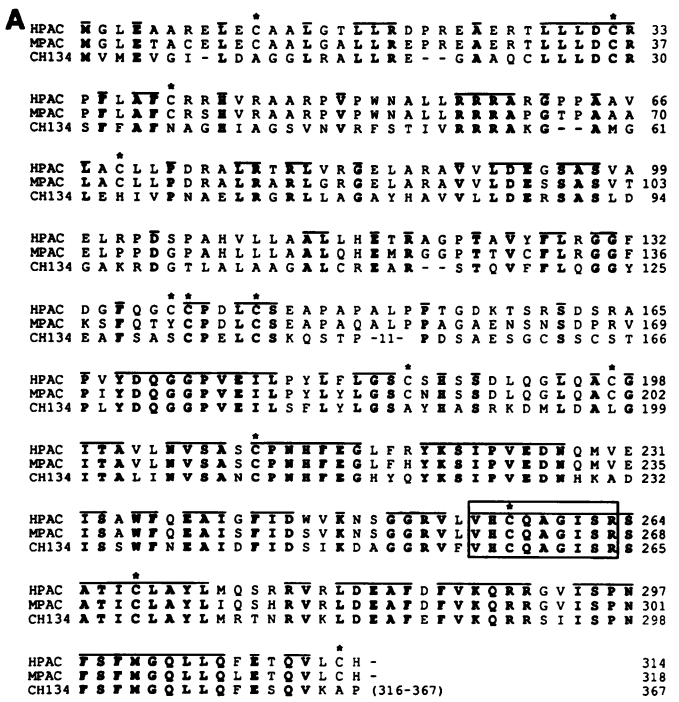

Fig. 1. Predicted amino acid sequence for human PAC-1 (HPAC) cDNA and comparison to similar proteins (*8*). Two independent, nearly full-length cDNA clones were isolated from a Lambda ZAP (Stratagene, La Jolla, California) cDNA library of oligo(dT)-primed mRNA derived from human PBT cells stimulated with PHA (1 μg/ml) and PMA (10 ng/ml) for 4 hours in the presence of cycloheximide (10 μg/ml). (**A**) The HPAC protein sequence compared to murine PAC-1 (MPAC) and murine 3CH134 (CH134). Amino acids conserved in all three proteins are in bold and overlined. Cysteine residues in HPAC are marked by asterisks; the HC motif is boxed. (**B**) Protein sequence comparisons of similar regions of HPAC, 3CH134, DAL1-A, and the VH1 and PTP-1B (*20*) phosphatases. Amino acid identities with PAC-1 are shown in bold. Dashes are inserted to allow for maximal alignment of the sequences. The consensus sequence (marked with asterisks) refers to HPAC, 3CH134, DAL1-A, and VH1 where four of four matches are shown in uppercase letters, and three of four matches are shown in lowercase letters.

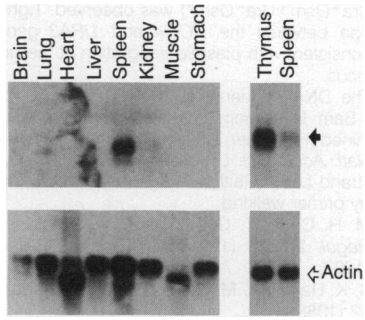

Fig. 2. Expression of PAC-1 in mouse tissues. All tissues were from adult BALB/c mice except thymus, which was from a 4-week-old mouse. Polyadenylated RNA (10 μg) was analyzed with a Northern blot (*3*) and a full-length murine PAC-1 cDNA insert probe or a murine β-actin insert probe (*21*). The two spleen RNA samples are equivalent and differ only in the relative exposure time of the blots. PAC-1 RNA is indicated by the solid arrow.

PTPases and that contains a cysteinyl residue that is required for activity (*6*, *7*). Within this region, the sequence HCX-AGXXR (referred to as an HC motif, where X is any amino acid) (*8*) is the minimal active site sequence for a large class of tyrosine-specific phosphatases. The conserved cysteine is thought to function as a phosphothiol intermediate (*9*).

A serum-inducible gene in murine 3T3 cells (*3CH134*) (*10*) and its human homolog (*CL100*) (*11*) encode proteins that are extensively similar throughout the entire length of PAC-1, with the highest degree of similarity (approximately 80%) within the COOH-terminal half (Fig. 1, A and B). Recombinant CL100 protein displays sodium vanadate–inhibitable activity toward *p*-nitrophenol phosphate (pNPP), a property of known PTPases (*11*). Another gene (*DAL1-A* for adjacent to *DAL1*), isolated from *Saccharomyces cerevisiae*, encodes a protein of unknown function adjacent to the allantoinase (*DAL1*) gene (*12*) and is similar to the PAC-1 protein over a stretch of 60 amino acids beginning just NH_2-terminal to the conserved HC motif and extending to the PAC-1 COOH-terminus (Fig. 1B). PAC-1 is similar to the vaccinia virus H1 (VH1) PTPase, an unusual phosphatase that has both tyrosine and serine phosphatase activity (*13*). The highest degree of similarity is within a 21–amino acid stretch that includes and extends COOH-terminal to the HC motif (Fig. 1B). PAC-1, 3CH134, DAL1-A, and VH1 have conserved, relative to other PTPases, the sequence (L or V)AYL(M or I) (*8*) at a distance 12 amino acids COOH-terminal to the active site cysteine. The presence of a tyrosine within this sequence is notable and suggests possible regulation through phosphorylation. Outside the HC motif, the PAC-1, 3CH134, and DAL1-A proteins are not similar to several other PTPases, which are exemplified by the PTP-1b phosphatase (*6*). PAC-1, 3CH134, and DAL1-A may constitute a new structural family of PTPases that also share regulatory or enzymatic properties with VH1. Bacterial recombinant PAC-1 protein has a relatively low catalytic rate of hydrolytic activity for pNPP (*14*). A small amount of activity could reflect a high degree of substrate specificity or, alternatively, a predominantly inactive tertiary structure of the protein produced in bacteria.

To investigate the tissue distribution of PAC-1, we performed Northern (RNA) analyses with polyadenylated RNA from several mouse tissues. A large amount of *PAC-1* expression was observed in RNA extracted from spleen and thymus tissue, but expression was undetectable in brain, lung, heart, liver, kidney, muscle, and stomach tissue (Fig. 2). Consistent with this organ distribution, *PAC-1* RNA was seen also in human bone marrow and tumor cell lines of pre-B, B, plasma, T, and myeloid cell origin (*15*). However, small amounts of *PAC-1* RNA have been observed in serum-stimulated human embryonic lung fibroblasts (*4*) and in the epithelial carcinoma cell line HeLa (*15*), which suggests that PAC-1 expression, although not strictly exclusive with regard to cell type, is comparatively high in hematopoietic cells. By contrast, 3CH134 is expressed in the largest amounts in lung tissue and is not detectable in spleen tissue (*10*), which suggests cell type–specific functions for PAC-1 and 3CH134.

Several polyclonal rabbit antisera were raised against PAC-1 peptides and against a recombinant bacterial glutathione-S-trans-

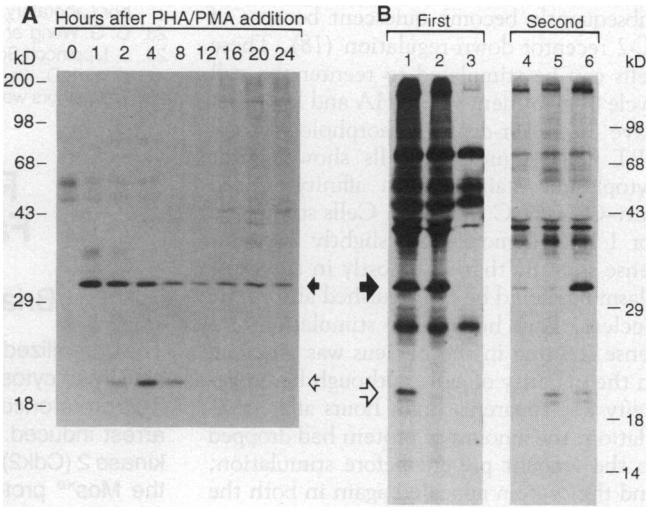

Fig. 3. Characterization of PAC-1 protein (**A**) Time course of synthesis of PAC-1 in activated PBT cells. T cells were enriched by passage of peripheral blood mononuclear cells over nylon wool (*4*) and subsequently stimulated (2×10^6 cells per milliliter) for various periods of time in RPMI 1640 medium that contained fetal calf serum (10%) with PHA (1 μg/ml) and PMA (40 ng/ml). One hour before harvesting, 2×10^8 cells were labeled with 200 μCi each of [^{35}S]methionine and [^{35}S]cysteine. Cells were lysed in a solution containing 1% NP-40, 150 mM NaCl, 50 mM EDTA, phenylmethylsulfonyl fluoride (100 μg/ml), and aprotinin (1 μg/ml). Extracts were cleared of nonspecifically absorbed proteins by incubation with protein A agarose (PAA) before incubation with antibodies. Extracts (5×10^5 cpm for 0, 1, and 2 hours and 19×10^5 cpm for the other times) were immunoprecipitated with affinity-purified anti(279–291) and PAA at 4°C for 2 hours. The NET–gel buffer system was used for precipitation and washing (*22*). Washed beads were analyzed on denaturing polyacrylamide gel electrophoresis (10% gel). Gels were impregnated with Enlightning (DuPont NEN Research Products, Boston, Massachusetts), dried, and exposed to film. PAC-1 and the 20-kD proteins are indicated by solid and open arrows, respectively. (**B**) Sequential precipitation of PAC-1 and a 20-kD cross-reactive protein. T cells were prepared and metabolically labeled as described above after stimulation with PHA and PMA for 3 hours. Lysates were immunoprecipitated at 4°C for 24 hours with PAA and the following antibodies: (i) anti(279–291); (ii) anti–GST-PAC (an affinity-purified rabbit polyclonal antibody to a recombinant GST fusion protein including HPAC amino acids 82 through 176); and (iii) an affinity-purified rabbit polyclonal antibody directed against an irrelevant peptide; the immunoprecipitated proteins are shown in lanes 1 to 3, respectively. The supernatants from the immunoprecipitation reactions shown in lanes 1 to 3 were subjected to a second immunoprecipitation with anti(279–291) and PAA at 4°C for 24 hours; the immunoprecipitated products are shown in lanes 4 to 6, respectively. All immunoprecipitates bound to PAA were treated as described above. PAC-1 and the 20-kD proteins are indicated by solid and open arrows, respectively.

ferase (GST) fusion protein (*16*). Several distinct antibodies specifically immunoprecipitated a 32-kD protein from extracts of [^{35}S]methionine-labeled human PBT cells stimulated for 3 hours with PHA and PMA. The relative synthesis of PAC-1 was highest within the first 4 hours after mitogenic stimulation of PBT cells and was thereafter observed at lower levels throughout the G1 phase of the cell cycle (Fig. 3A). Consistent with this, the amount of PAC-1 RNA peaked at approximately 2 hours, and no RNA was detectable by 24 hours after stimulation (*3*, *4*). In addition, antisera to a single peptide encompassing amino acids 279 to 291 [anti(279–291)], a highly conserved region between PAC-1, 3CH134, and DAL1-A, precipitated with PAC-1 a 20-kD mitogen-inducible protein that was transiently synthesized approximately 1 to 8 hours after stimulation (Fig. 3A). Sequential precipitation of labeled extracts show that PAC-1 and the 20-kD protein share a cross-reactive epitope. PAC-1 could be removed by antiserum to the GST fusion protein (anti–GST-PAC), and the 20-kD protein could be subsequently precipitated with the anti(279–291) serum (Fig. 3B).

The subcellular location of PAC-1 was investigated by immunofluorescent staining with affinity-purified antibodies. COS-7 cells transiently transfected with a PAC-1 expression vector demonstrated positive staining on approximately 10% of the cells. Positively stained cells showed a uniform pattern of strong punctate staining in the nucleus with no staining of the nucleolus (Fig. 4A). Diffuse staining in the cytoplasm above the background intensity of nontransfected cells was observed. Similar results were obtained with an epitope-tagged version of PAC-1 and monoclonal antibodies to the tag (*17*).

The nuclear location of PAC-1 was confirmed by the localization of endogenously synthesized PAC-1 in untreated or mitogen-stimulated cells. Immunofluorescence was done on a polyclonal human T cell line that had been stimulated by PHA, expanded by growth in interleukin-2 (IL-2), and

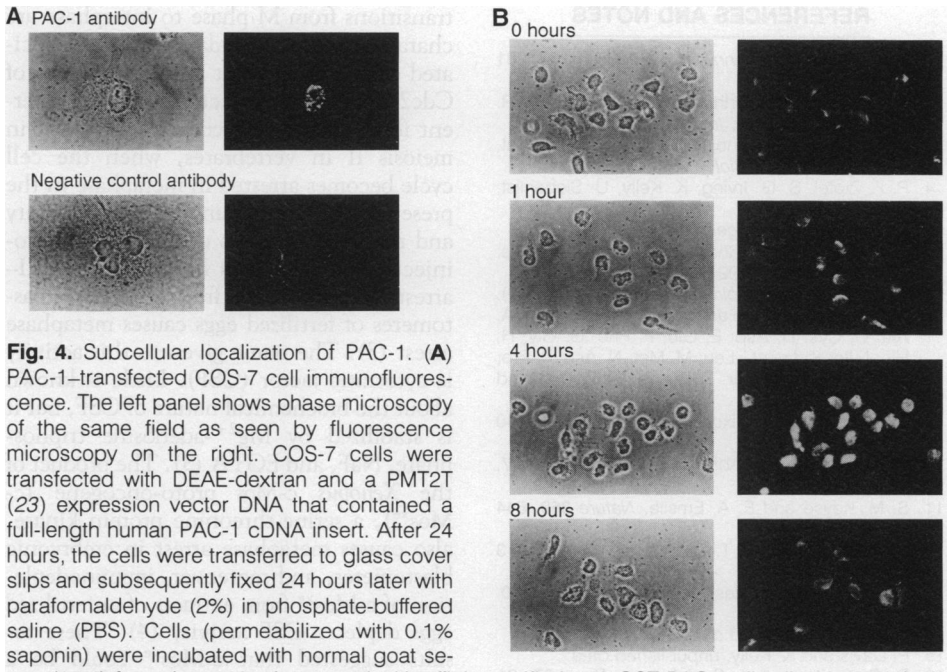

Fig. 4. Subcellular localization of PAC-1. (**A**) PAC-1–transfected COS-7 cell immunofluorescence. The left panel shows phase microscopy of the same field as seen by fluorescence microscopy on the right. COS-7 cells were transfected with DEAE-dextran and a PMT2T (*23*) expression vector DNA that contained a full-length human PAC-1 cDNA insert. After 24 hours, the cells were transferred to glass cover slips and subsequently fixed 24 hours later with paraformaldehyde (2%) in phosphate-buffered saline (PBS). Cells (permeabilized with 0.1% saponin) were incubated with normal goat serum (15%) for at least 10 min, treated with affinity-purified anti–GST-PAC antibodies (1:250) for 1 hour, washed with PBS that contained bovine serum albumin (2%) and saponin (0.1%), incubated with fluorescein isothiocyanate–labeled goat antibodies [$F(ab')_2$ fragments] to rabbit immunoglobulin G (1:100) for 20 min, washed as above, and mounted with Fluoromount-G (Southern Biotechnology Associates, Birmingham, Alabama). Control antibodies were prebleed normal rabbit serum (above) or affinity-purified antibodies to GST. (**B**) Immunofluorescence of mitogen-activated T cells. The left and right panels are phase and fluorescence microscopy of the same fields, respectively. T cells were collected at the indicated times after stimulation with PHA (1 μg/ml) and PMA (40 ng/ml), adhered to concanavalin A–coated cover slips (*24*), and fixed and stained as described above.

subsequently become quiescent because of IL-2 receptor down-regulation (*18*). These cells can be stimulated to reenter the cell cycle by treatment with PHA and PMA yet have a better-defined morphology than PBT cells. Quiescent cells showed faint cytoplasmic staining with affinity-purified anti–GST-PAC (Fig. 4B). Cells stimulated for 1 hour demonstrated slightly more intense staining that was mostly in the cytoplasm but could be distinguished also in the nucleus. Four hours after stimulation, intense staining in the nucleus was observed in the majority of cells, although heterogeneity was apparent. Eight hours after stimulation, the amount of protein had dropped to the amount present before stimulation, and the protein appeared again in both the cytoplasm and the nucleus.

A nuclear tyrosine phosphatase might act on any number of potentially important substrates, including phosphoproteins that regulate cell cycle progression or transcription. It recently has been demonstrated that tyrosine phosphorylation regulates the localization and activity of the transcription factor complex that mediates interferon-α–induced gene expression (*19*). Tyrosine phosphorylation and dephosphorylation of nuclear proteins may be a generalized mechanism for signal transduction stimulated by other ligands or physiological situations.

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Requirement for Cdk2 in Cytostatic Factor–Mediated Metaphase II Arrest

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The unfertilized eggs of vertebrates are arrested in metaphase of meiosis II because of the activity of cytostatic factor (CSF). *Xenopus* CSF is thought to contain the product of the *Mos* proto-oncogene, but other proteins synthesized during meiosis II are also required for arrest induced by CSF. In *Xenopus* oocytes, ablation of synthesis of cyclin-dependent kinase 2 (Cdk2) during meiosis resulted in absence of the metaphase II block, even though the Mos^{xe} protein kinase was fully active at metaphase. Introduction of purified Cdk2 restored metaphase II arrest, and increasing the amount of Cdk2 during meiosis I (when Mos^{xe} is present) led to metaphase arrest at meiosis I. These data indicate that metaphase arrest is a result of cooperation between a proto-oncogene kinase and a cyclin-dependent kinase and illustrate the interaction of a cell growth regulator with a cell cycle control element.

Recent progress in the study of cell cycle control has established that the regulation of both meiosis and mitosis (M phase) in eucaryotic cells is controlled by the activity of the Cdc2 protein kinase (*1*). When active, Cdc2 is complexed with a specific form of cyclin molecule, and cell cycle transitions from M phase to interphase are characterized by degradation of the associated cyclin component and inactivation of Cdc2 protein kinase activity (*2*). A different form of M phase control is evident in meiosis II in vertebrates, when the cell cycle becomes arrested in metaphase in the presence of high amounts of Cdc2 activity and maximal amounts of cyclin B. Microinjection of cytoplasm from metaphase II–arrested, unfertilized frog eggs into blastomeres of fertilized eggs causes metaphase arrest (*3*). The name given to this activity is cytostatic factor (CSF). Little is known about the biochemical nature of CSF, but it is stabilized by Mg^{2+}-adenosine triphosphate, NaF, and EGTA (*3*). The product of the *Xenopus* c-*Mos* proto-oncogene (c-Mos^{xe}), a serine-threonine protein kinase, also causes metaphase arrest in embryonic blastomeres, and moreover, immunodepletion of c-Mos^{xe} from extracts of unfertilized eggs depletes CSF activity (*4*). These results suggested that c-Mos^{xe} could be a component of CSF. Upon fertilization of eggs, the intracellular concentration of free Ca^{2+} increases, cyclin is rapidly degraded, followed, more slowly, by the disappearance of the c-Mos^{xe} protein that migrates with a slower electrophoretic mobility (*5*). These results suggest that the loss of CSF activity at fertilization involves changes in the expression of c-Mos^{xe}.

Protein synthesis is required for both meiosis I and meiosis II in *Xenopus* and mice (*6*), and the ability of recombinant c-Mos^{xe} to induce meiosis I in *Xenopus* oocytes has been taken as evidence that the sole protein synthesis requirement for meiosis I is accounted for by the new synthesis of c-Mos^{xe} (*7*). However, meiosis II in *Xenopus* is not stimulated by the active c-Mos^{xe} kinase, suggesting that other newly synthesized components are also required (*7*). One possible component that could account for the additional protein synthesis requirement in meiosis II is the product of the cyclin-dependent kinase 2 gene (*Cdk2*) in *Xenopus*. The mRNA encoding Cdk2 is newly polyadenylated in oocytes stimulated to enter meiosis I by addition of progesterone, and after fertilization, the Cdk2 mRNA is deadenylated, suggesting that, like c-Mos^{xe}, the Cdk2 protein kinase functions in the meiotic cell cycles of oocytes (*8*).

In *Xenopus* embryos Cdk2 protein kinase activity is evident only in a complex of large molecular size, and is higher in M phase than in interphase (*9*). The active kinase persists at least until gastrulation (*10*). During oocyte maturation, the activity of Cdk2 is low at germinal vesicle breakdown (GVBD) in meiosis I and increases by meiosis II (*9*). In embryonic or somatic cell cycles Cdk2 may also function in G_1 in regulating entry into S phase (*11*) or in the mechanism coupling activation of

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