Interaction of the Essential Drosophila Nuclear Protein YA with P0/AP3 in the Cytoplasm and in Vitro: Implications for Developmental Regulation of YA’s Subcellular Location

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The Drosophila nuclear lamina protein YA is essential for the transition from female meiosis to embryo mitosis. Its localization and, hence, function is under developmental and cell cycle controls. YA protein is hyperphosphorylated and cytoplasmic in ovaries. Upon egg activation, YA is partially dephosphorylated and acquires the ability to enter nuclei. Its function is first detected at this time. To investigate the cytoplasmic retention machinery that keeps YA from entering nuclei, we used affinity chromatography and blot overlay assays to identify cytoplasmic proteins that associate with YA. Drosophila P0/AP3, a ribosomal protein that is also an apurinic/apyrimidinic endonuclease, binds to YA in ovary and embryo cytoplasm. P0 and YA bind specifically and directly in vitro and are present in a 20S complex in the cytoplasmic extracts. YA protein can be phosphorylated by MAPK, but not by p34 Cdc2 kinase, in vitro. This phosphorylation increases YA’s binding to P0. We propose that the P0-containing 20S cytoplasmic complex retains hyperphosphorylated ovarian YA in the cytoplasm. In response to egg activation, YA is partially dephosphorylated and its binding to the 20S complex is reduced. Hence, some YA dissociates from the complex and enters nuclei. Consistent with this model, decreasing P0 levels partially suppress a hypomorphic Ya mutant allele.

Key Words: Drosophila; P0/AP3; YA; MAPK; egg activation; nuclear entry; phosphorylation.

INTRODUCTION

Oocyte growth, maturation, and activation are critical processes for successful production of an embryo. During oogenesis, oocytes stockpile mRNAs and proteins that will be used subsequently for embryo development. During oocyte maturation, oocytes acquire the capability to be activated and fertilized. Mature oocytes in all vertebrates and many invertebrates, including Drosophila, arrest during meiotic metaphase awaiting egg-activation signals. Egg activation triggers completion of meiosis, allowing the formation of a haploid female pronucleus that can participate in further development. In several organisms, the phosphorylation states of some maternal proteins undergo changes that are important for proper progression through oocyte maturation, metaphase arrest, and egg activation. For example, inhibition of phosphorylation of maternal proteins during meiotic metaphase arrest can activate Xenopus eggs (Zhang and Masui, 1992), suggesting that phosphorylation of maternal proteins is essential to maintain the meiotic metaphase arrest.

Maturation promoting factor (MPF; p34 Cdc2 kinase and cyclin B complex) and mitogen-activated protein kinase (MAPK) are two key kinases during these processes. In most invertebrates and vertebrates (e.g., the bivalve Ruditape, ascidians, Xenopus, mouse), MPF and MAPK activities increase during oocyte maturation, remain high during oocyte metaphase arrest, and decrease after initiation of egg activation (Colas and Guerrier, 1995; Russo et al., 1998). MPF activity is required for oocyte maturation and the maintenance of meiotic arrest, and its inactivation is essential for egg activation (Colas and Guerrier, 1995; Palmer and Nebra, 2000). MPF is responsible for chromatin condensation and nuclear envelope disassembly during Xenopus metaphase arrest (Lohka, 1998). In most organisms, MAPK

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is not required for germinal vesicle breakdown (GVBD) or MPF activation during oocyte maturation, but is required for the maintenance of oocyte metaphase arrest (Fisher et al., 1999; Gross et al., 2000; Kosako et al., 1994a,b). In Xenopus, a transient early high level of MAPK is required for the correct timing and efficiency of GVBD, and MAPK increases MPF activity (Fisher et al., 1999; Gross et al., 2000). MAPK is required to suppress entry into S phase between meiosis I and meiosis II (Gross, 2000). MAPK is also sufficient for MPF activation at the onset of oocyte maturation (Gotoh et al., 1995; Yew et al., 1995; Huang et al., 1995). The situation in some other organisms, such as Chaetopterus, is a bit different; MAPK is neither required nor sufficient for oocyte maturation or meiotic arrest (Eckberg, 1997). In mouse, high MAPK activity during oocyte maturation and metaphase arrest is important for the maintenance of chromosome condensation and the metaphase block, formation of the meiotic spindles, and prevention of pronuclear envelope formation (Choi et al., 1996; Moos et al., 1995; Verlhac et al., 1994). The phosphorylation targets of MAPK that execute these functions are largely unknown, with the exception of p90(23), which is phosphorylated by MAPK during Xenopus oocyte maturation (Bhatt and Ferrell, 1999; Gavin et al., 1999; Gross et al., 1999; Grove et al., 1993; Sturgill et al., 1988). The role or modulation of MPF and MAPK in Drosophila oocytes is as yet unknown. Drosophila oocytes arrest at meiotic prophase I during most of oogenesis. Toward the end of oogenesis, they resume meiosis, only to arrest again at metaphase I (Page and Orr-Weaver, 1997). Egg activation occurs independent of and prior to fertilization (Doane, 1960; Hefetz et al., 2001).

The YY (Young Arrest) phosphoprotein is essential for nuclear events during the developmental transition from female meiosis to embryo mitosis (Lin and Wolfner, 1991; Liu et al., 1995; Yu et al., 1999). Embryos from females lacking YY function arrest just after completing meiosis. Zygotic mitosis never occurs in these embryos (Lin and Wolfner, 1991; Liu et al., 1995; Lopez, 1996; Berman, 2000). In normal embryos and activated oocytes, YY is found in interphase through metaphase nuclei, in the nuclear lamina layer of the nuclear envelope, where it associates with lamin (Goldberg et al., 1998), and in the nucleoplasm, where it associates with chromatin (Lopez and Wolfner, 1997) through its direct interaction with DNA and with histone H2B (Yu and Wolfner, 2002). There is also a cytoplasmic pool of YY throughout the cell cycle. In oocytes, YY is in a more highly phosphorylated form and is excluded from oocyte nuclei, and away from chromatin (Yu et al., 1999). Thus, only after egg activation does YY acquire its ability to enter nuclei, coincident with its partial dephosphorylation. Based on this correlation, we proposed that phosphorylation regulates YY’s potential for nuclear entry during development (Yu et al., 1999).

YY contains five predicted MAPK target sites and four predicted p34(Cdc)2 kinase target sites (Liu and Wolfner, 1998). Thr, which is both a p34(Cdc)2 kinase and MAPK predicted target site, is one of at least two sites in YY that are differentially phosphorylated during this developmental transition (Yu et al., 1999). This suggests that phosphorylation of YY by one or both of these kinases may regulate YY’s subcellular location.

Phosphorylation can regulate a protein’s nuclear entry by modulating the protein’s interaction with cytoplasmic retention machinery (Jans and Hubner, 1996). In one phosphorylation state, the target protein binds the cytoplasmic retention machinery and stays in the cytoplasm. In another phosphorylation state, the protein loses affinity for the cytoplasmic retention machinery and can enter nuclei via its nuclear localization signals (NLS). This type of regulation is typified by the nuclear import of the Drosophila Dorsal protein (Drier et al., 1999; Liu et al., 1997; Whalen and Steward, 1993). Dorsal is retained in the cytoplasm by binding to Cactus protein. In response to dorsoventral signaling, Dorsal and Cactus are both phosphorylated, causing Dorsal to dissociate from Cactus and leading to Dorsal’s nuclear entry.

Here, we present evidence that the changes in YY’s subcellular localization may utilize an analogous mechanism, but one regulated during egg activation. We report that YY binds to P0/AP3, a Drosophila ribosomal protein which also has apurinic/apyrimidinic endonuclease activity (Yacoub et al., 1996). In vitro, this interaction is influenced by YY’s phosphorylation state: YY is a target of MAPK (but not p34(Cdc)2 kinase) in vitro, and MAPK-phosphorylated YY binds more strongly to P0 than YY that was not phosphorylated by MAPK. We propose that MAPK phosphorylation of YY in ovaries results in YY’s retention in the cytoplasm within a 20S complex that also contains P0. Upon activation, dephosphorylation of YY releases it from the complex, allowing it to enter nuclei. Consistent with this model, lowering the levels of P0 partially suppresses a hypomorphic YY mutant allele. YY provides a molecular probe with which to assess the cause and consequences of phosphorylation changes during Drosophila egg activation.

**MATERIALS AND METHODS**

**Production, Purification, and Coupling of Recombinant Proteins**

To make a protein fusion of YY to a tag [E. coli maltose-binding protein (MBP)] and to allow labeling in vitro [a heart-muscle kinase (HMK) target site (Blanar and Rutter, 1992)], full-length YY cDNA (Lopez et al., 1994) was cloned into a modified pMALTM-c2 vector (NEB) called pMALC2.HMK(R) (Z. Li and M. L. Goldberg, personal communication). The fusion protein is called MBP-HMK-YY. A full-length P0 cDNA (Yacoub et al., 1996) was cloned into pET21a(-) vector (Novagen) to express P0-His fusion protein.

Protein induction and expression were done according to NEB’s protein fusion and purification (pMALTM) instruction manual (for MBP-HMK-YY) or the PET system manual (Novagen) (for P0-His) with the following modifications. The construct was transformed
into Escherichia coli BL21(DE3)plysS cells (for MBP-HMK-YA) or BL21(DE3) (for P0-His). An overnight culture was diluted 1:20 into fresh medium. IPTG was added to a final concentration of 1 mM (for MBP-HMK-YA) or 0.4 mM (for P0-His). All the following steps after harvest of cells were performed at 4°C. The cell pellet was immediately resuspended and sonicated. Triton X-100 was added to the cell lysate to a final concentration of 1%. The lysate was incubated for 1 h with continuous mixing, and centrifuged at 39,000g for 30 min. MBP-HMK-YA was purified from the supernatant with amylose beads, and P0-His was purified with His-bind beads (Novagen). The peak fractions were pooled and analyzed by 7.5% SDS-polyacrylamide gels with Coomassie blue staining and Western blotting with affinity-purified anti-YA or anti-P0 antibodies.

Like endogenous YA (Liu and Wolfrer, 1998), purified MBP-HMK-YA protein can interact with embryonic YA (data not shown). MBP-HMK-YA can also be incorporated into the nuclear envelope of in vitro assembled nuclei in Xenopus egg extracts (M.F.W., unpublished observations), consistent with its being capable of at least some of YA’s normal behaviors.

A total of 4 mg of MBP-HMK or MBP-HMK-YA was coupled to 1 ml CNBr-activated Sepharose-4B beads (Sigma) following the method of Goldberg et al., 1998. The pGBT9-YA constructs were cotransformed with pGAD424 (negative control), pGAD424-P0, or pGAD424-lamin Dm0 (positive control; Goldberg et al., 1998) into yeast strains HF7c and SFY526. The β-gal activity was assayed qualitatively (by color of the colonies; HF7c strains) and quantitatively (for β-Gal activity units; SFY526 strains) as described in Goldberg et al. (1998). Each combination was tested in duplicate. β-Gal units are the average of three determinations per experiment; data from one experiment per combination is shown.

Immunofluorescence staining

Embryos at 0–2 h were collected and processed as described in Lin and Wolfrer (1991). Embryos were incubated with affinity-purified anti-P0 antibodies (1:50). Anti-P0 antibodies were purified from crude antisera kindly provided by M. R. Kelley (Yacoub et al., 1998) by using a GST-P0 protein-coupled Sepharose 4B column. The embryos were then stained with FITC-conjugated anti-rabbit IgG antibodies (1:100) and counterstained with DAPI. Images were collected by using laser scanning confocal microscopy (Bio-Rad MRC 600 system on a Zeiss inverted microscope).

Blot Overlay Assay

To make probes, 2 μg of MBP-HMK-YA or MBP-HMK was incubated in a 100-μl labeling reaction and incubated at room temperature for 3 h (Blanar and Rutter, 1992). Labeling of proteins was verified by SDS-PAGE followed by autoradiography. Proteins to be analyzed by blot overlay assay were separated by SDS-PAGE and transferred to nitrocellulose membranes as described in Lin and Wolfrer (1991). The blot overlay assay was performed as Blanar and Rutter (1992) with minor modifications.

Protein Sequencing

Proteins for sequencing were separated on SDS-PAGE and transferred to PVDF membranes according to Lin and Wolfrer (1991). The membrane was stained with Coomassie blue and destained with milliQ water. The band of interest was cut from the membrane and sequenced from the protein’s N terminus with a PE/Applied Biosystems Precise 492, by the Cornell University BioResource Center. The unambiguous amino acids in the peptide sequence thus obtained were used to search the nr database to identify the protein.
were then harvested and washed with 1 × Hyb(75) with 0.05% NP-40 eight times. Proteins were eluted from the beads by boiling in SDS-PAGE sample buffer. One-twentieth of the supernatant after binding and half the eluate from the beads were analyzed by Western blotting with affinity-purified anti-P0 antibodies and anti-YA antibodies.

For coimmunoprecipitation with anti-YA antibodies, 2 μl of affinity-purified anti-YA antibodies (Lin and Wolfner, 1991) were added to cytoplasmic extracts from 5 μl of packed 0- to 2-h embryo from wild-type or Ya2 mutant females. Presence of YA and P0 in samples corresponding to 1 μl of embryos was assessed by Western blotting; supernatant after binding and the eluate from the beads were examined.

Sucrose Density Gradient Sedimentation Assay

Embryos at 0-2 h were homogenized in 4 vol of buffer D (50 mM Tris-HCl, pH 7.5, 50 μM MgCl2, 145 mM sucrose) with 100 mM NaCl and 0.25% Triton X-100. The supernatant was loaded onto a 5–35% sucrose density gradient in the same buffer. Centrifugation conditions were as described in Swanson and Vacquier (1997). The collected fractions were precipitated with 2 vol of acetone. The pellet was dissolved in 1 × SDS-PAGE sample buffer, boiled for 5 min, and analyzed by Western blotting. All steps were performed at 4°C. Molecular weight markers (MW-GF-1000; Sigma) were run in each experiment. The sedimentation assay was performed according to Resnick et al. (1997). All buffers following centrifugation were performed according to Resnick et al. (1997). All buffers following centrifugation were performed according to Resnick et al. (1997) three times and with 1 × kinase buffer and 100 μM ATP and 20 Ci/mmol [γ-32P]ATP (final concentration). MBP-HMK-YA (4 μg) was incubated in a final volume of 100 μl containing 1 × kinase buffer and 200 μM ATP with or without (mock phosphorylation) 14 μl immunoprecipitated p34(Cdc2) kinase and/or 4 μl MAPK. [γ-32P]ATP was added to a 10-μl aliquot of the above reaction mix to a final concentration of 20 mCi/μmol. All buffers were supplemented with protease inhibitors and phosphatase inhibitors (25 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin). All the reactions were incubated at room temperature for 4 h with rotation. The reactions with radioactive ATP were analyzed by SDS-PAGE. Reactions with nonradioactive ATP were used for MBP pull-down assays.

MBP Pull-Down Assay

To test the specificity of the cytoplasmic proteins’ binding to YA, extracts from 10 μl of packed 0- to 2-h embryos were incubated overnight with 20 μl of either MBP-HMK-YA beads or MBP-HMK-YA beads in the absence or presence of 2 μg MBP-HMK or MBP-HMK-YA. The beads were then washed with 1 × Hyb(75) + 0.05% NP-40 eight times. Proteins bound to the beads were eluted with 1 × Hyb(75) + 0.05% NP-40 + 1 M NaCl. To test purified P0’s direct binding to YA, 2 μg of purified MBP-HMK-YA or 2 μg of purified MBP-HMK were incubated with 4 μg of P0-His6 in 1 × Hyb(75) + 0.05% NP-40 with protease inhibitors (1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin) for 1 h. Amylose beads (30 μl) were then added to the solution, which was then rotated end-to-end for 5 h. After centrifugation, the beads were washed and harvested with four times with 1 ml of 1 × Hyb(75) + 0.05% NP-40 + 200 mM NaCl with protease inhibitors. The beads were eluted by boiling in 4 × SDS-PAGE sample buffer for 5 min. The supernatant after the binding was precipitated with 2 vol of acetone at −20°C overnight. The pellet was dissolved in 1 × SDS-PAGE sample buffer and boiled for 5 min. The supernatant after binding and half of the eluate from the beads were analyzed by SDS-PAGE followed by Western blotting with affinity-purified anti-P0 antibodies. All steps were performed at 4°C.

To test the effect of phosphorylation of YA on YA’s interaction with P0, different dilutions of in vitro phosphorylated YA and mock phosphorylated YA (0.7 or 0.3 μg) were mixed with 3 μg P0-His6 in a final volume of 250 μl containing 1 × Hyb(75), 0.05% NP-40, and protease inhibitors. The binding reaction was performed as above.

**Immunoprecipitation of p34<sup>Cdc2</sup> Kinase from Hela Cells**

Cell culture, synchronization, and lysis of Hela S3 cells were performed according to Resnick et al. (1997). All buffers following cell lysis were supplemented with protease inhibitors and phosphatase inhibitors (25 mM NaF, 1 mM Na vanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Anti-p34<sup>Cdc2</sup> kinase antibodies (10 μg/ml) were added to cell lysates containing about 500 μg protein and incubated for 2 h at 4°C. Protein A-Sepharose CL-4B beads (20 μl; Sigma) were then added and the mixture was rotated for 1 h at 4°C. The beads were harvested and washed once with KLB (Resnick et al., 1997) three times and with 1 × kinase buffer (NEB). The beads were then resuspended in 50 μl 1 × kinase buffer and stored at 4°C.

**In Vitro Phosphorylation Reactions**

MBP-HMK-YA fusion protein (0.5 μg) or 1 μg histone H1 in 1 × kinase buffer was incubated with 2 μl immunoprecipitated p34<sup>Cdc2</sup> kinase or 0.5 μl MAPK (ERK2, NEB) in a final volume of 25 μl containing 1 × kinase buffer and 100 μM ATP and 20 Ci/mmol [γ-32P]ATP (final concentration). MBP-HMK-YA (4 μg) was incubated in a final volume of 100 μl containing 1 × kinase buffer and 200 μM ATP with or without (mock phosphorylation) 14 μl immunoprecipitated p34<sup>Cdc2</sup> kinase and/or 4 μl MAPK. [γ-32P]ATP was added to a 10-μl aliquot of the above reaction mix to a final concentration of 20 mCi/μmol. All buffers were supplemented with protease inhibitors and phosphatase inhibitors (25 mM NaF, 1 mM Na vanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin). All the reactions were incubated at room temperature for 4 h with rotation. The reactions with radioactive ATP were analyzed by SDS-PAGE. Reactions with nonradioactive ATP were used for MBP pull-down assays.

**Genetic Suppression Analysis**

P0<sup>306</sup>/M KR5 stock was kindly provided by M. V. Frolov and J. A. Birchler (Frolov and Birchler, 1998). X; X, y Ya<sup>17</sup> cv v f/Y, Ya<sup>y</sup> y w<sup>B</sup> (Liu et al., 1995) virgin females were mated with P0<sup>306</sup>/M KR5 males. Then, 0- to 2-h embryos were collected from X; X, y Ya<sup>17</sup> cv v f/Y, P0<sup>306</sup>× +, and X; X, y Ya<sup>17</sup> cv v f/Y, +/M KR5 females, respectively. All embryos were stained with DNA dye DAPI and examined with an Olympus BX50 equipped with a Pentax camera (Princeton Instruments). Three independent repeats of the experiment gave comparable results. The results of one of those experiments is shown.

**RESULTS**

**Drosophila P0 Interacts with YA**

To identify cytoplasmic proteins that interact with YA and could serve as YA’s cytoplasmic retention machinery, we employed a blot overlay assay to detect proteins that interact with YA directly, and affinity chromatography to purify those proteins. The bands from ovary and syncytial-stage embryo cytoplasmic samples were detected by a 32P-labeled MBP-HMK-YA probe (data not shown), suggesting that the same group of proteins from ovary and embryo cytoplasm interact with YA. As it is easier to get
large amounts of embryos for protein purification, embryos rather than ovaries were used as starting material to purify YA-interacting proteins from cytoplasm.

We then tested whether the proteins that bound to the 32P-labeled MBP-HMK-YA probe in the blot overlay assay could bind to MBP-HMK-YA specifically and in a nondenatured condition suitable for affinity column purification. Cytoplasmic extracts from 0- to 2-h embryos were incubated with MBP-HMK-YA-coupled beads or MBP-HMK-coupled beads in the presence or absence of excess amounts of uncoupled MBP-HMK-YA or MBP-HMK protein (see Material and Methods). Proteins bound to the beads were eluted and analyzed by blot overlay assay. As shown in Fig. 1 of the blot probed with 32P-labeled MBP-HMK-YA probe, in the presence of excess unlabelled MBP-HMK, six proteins that bind to MBP-HMK-YA-coupled beads were detected by the probe (Fig. 1, lane 2). Of these, three proteins, P35, P12 and P10, did not bind to MBP-HMK-coupled beads (Fig. 1, lane 1), suggesting that they only bound to the YA moiety. P25 bound to MBP-HMK beads very weakly but to MBP-HMK-YA beads strongly, suggesting that it has weak nonspecific binding to MBP-HMK and/or the beads, but it has strong affinity for YA. Confirming this, the binding of P35, P25, P12, and P10 to MBP-HMK-YA-coupled beads was competed by MBP-HMK-YA in solution (Fig. 1, lane 3), but not by MBP-HMK in solution (Fig. 1, lane 4). The other two proteins, P42 and P30, bound to both MBP-HMK-YA-coupled beads and MBP-HMK-coupled beads (Fig. 1, lanes 1 and 2), suggesting that they could bind to the MBP-HMK, YA moiety, and/or the beads. Their binding to MBP-HMK-YA beads was competed by MBP-HMK-YA, but not MBP-HMK, in solution (Fig. 1, lanes 3 and 4), suggesting that they bound to both the YA moiety of MBP-HMK-YA and the beads. Since P42 and P30 do not bind to YA specifically, they were not studied further. The blot probed with 32P-labeled MBP-HMK probe in the presence of excess unlabelled MBP-HMK (Fig. 1) did not have any signal, suggesting that the blockage by the nonlabeled MBP-HMK of any potential 32P-labeled MBP-HMK probe’s binding to the proteins on the blot is complete; it also confirms that the signals from the blot probed with 32P-labeled MBP-HMK-YA came from YA’s binding to the proteins on the blot.

We used affinity chromatography to enrich the four proteins that interact with YA specifically (P35, P25, P12, and P10) (see Materials and Methods). We obtained enough P35 for N-terminal sequencing; we did not pursue the other three proteins further in this study, The N-terminal sequence of P35 was determined (Fig. 2A). Of the 20 amino acids that the Cornell sequencing facility was able to identify, 14 were unambiguous; there was uncertainty in 6 due to the limited amount of sample. The unambiguous sequence was used to search the Drosophila genome (Adams et al., 2000). There was only one match — Drosophila P0/AP3 (Fig. 2A), an apyrinic/apurimic endonuclease that is also a ribosomal protein (Yacoub et al., 1996); we will abbreviate this protein as P0. Four amino acids do not match the P0 genomic sequence reported by Adams et al. (2000); these mismatches are not due to strain differences based on genomic PCR from the strain we used for protein purification (data not shown); we therefore believe they are due to machine error given to the limited amount of protein available for sequencing. The first amino acid, Met, is in the
predicted P0 sequence but not in the sequence of P35. Since the P0 sequence is derived by conceptual translation from the cDNA, this suggests that this Met is cleaved from the protein after translation \textit{in vivo} (Bradshaw et al., 1998).

Four lines of evidence support the conclusion that P35 is \textit{Drosophila} P0. First, the predicted molecular weight of P0 (34 kDa) and the size of its \textit{in vitro} translation product (Kelley et al., 1989) are the same as the size of P35 (35 kDa). Second, affinity-purified anti-P0 antibodies (Yacoub et al., 1996) cross-react with a band that migrates at the same molecular weight as P35 and on Western blots parallels the distribution of P35 during purification (Fig. 2B). Third, purified recombinant P0 protein binds to YA directly \textit{in vitro} (Fig. 3). His6-tagged P0 was made and purified from E. coli and incubated with MBP-HMK-YA or MBP-HMK. Amylose beads were added to pull down the MBP-HMK fusion proteins (see Materials and Methods). P0-His6 was pulled down by amylase beads with MBP-HMK-YA, but not with MBP-HMK, indicating that P0 binds specifically to the YA moiety of MBP-HMK-YA. Finally, YA interacts with Drosophila P0 in the yeast two-hybrid system as seen with both qualitative and quantitative assays (Table 1). These data, taken together, indicate that the YA-interacting protein P35 is \textit{Drosophila} P0.

**P0 and YA Are Members of a Complex in Both Embryo and Ovary Cytoplasmic Extracts**

P0 is an abundant multifunctional protein that is expressed throughout \textit{Drosophila} development, and is reported to be found in both nuclear and cytoplasmic subcellular fractions (Yacoub et al., 1996). We confirmed that it is abundant in the cytoplasm of 0- to 2-h embryos (Fig. 4).

To further examine the interaction of YA and P0 in embryos and ovaries, we performed coimmunoprecipitation assays with embryo and ovary cytoplasmic extracts (Fig. 5A). In the presence of affinity-purified anti-P0 antibodies, YA was precipitated with P0 from both ovarian and embryo cytoplasm (Fig. 5A, lanes 2 and 4). Immunoprecipitation

![FIG. 2. P35 is Drosophila P0. (A) The N-terminal sequence of P35 purified from MBP-HMK-YA column aligned with the N-terminal sequence of Drosophila P0/AP3, a ribosomal protein that also has apurinic/apyrimidinic endonuclease activity (Yacoub et al., 1996). The residues in lowercase are those for which the sequencing facility reported uncertainty. (B) Western blot of embryo cytoplasmic proteins that passed through an MBP-HMK coupled column and were loaded onto a MBP-HMK-YA column (ft1), the flow through from the MBP-HMK-YA column (ft2), the wash (wash), and the eluate from the MBP-HMK-YA column with increasing concentrations of NaCl (elution). The fractions separated by SDSPAGE were probed with anti-P0 antibodies. These antibodies recognize P35, the 35-kDa band that bound to MBP-HMK-YA. The ft1 and ft2 lanes each contain 0.5 U of extracts (Fisher et al., 1982); the other lanes each contain 20 U of extracts.](FIG.2.jpg)

![FIG. 3. P0-His6 binds specifically to YA in vitro. A total of 2 μg of MBP-HMK (Tag-YA) or MBP-HMK (Tag) was incubated with 4 μg of P0-His6, and pulled down with amylase beads. All of the supernatant (Sup.) and half of the proteins bound to beads (Beads) were analyzed by Western blotting with anti-P0 antibodies. P0-His6 was pulled down with MBP-HMK-YA (lane 3) but not with MBP-HMK (lane 4).](FIG.3.jpg)

**TABLE 1**

Interaction of YA and P0 As Measured by the Yeast Two-Hybrid Assay

<table>
<thead>
<tr>
<th>Genes fused to pGBT9/</th>
<th>Genes fused to pGAD424</th>
<th>β-Gal color</th>
<th>β-Gal activity (U)</th>
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<tr>
<td>YA/none</td>
<td>All-A</td>
<td>White</td>
<td>0.00638</td>
</tr>
<tr>
<td>YA/P0</td>
<td>All-A</td>
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<td>0.184</td>
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<td>All-A</td>
<td>Blue</td>
<td>2.693</td>
</tr>
<tr>
<td>YA/none</td>
<td>All-E</td>
<td>White</td>
<td>0.141</td>
</tr>
<tr>
<td>YA/P0</td>
<td>All-E</td>
<td>Light blue</td>
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<tr>
<td>YA/lamin</td>
<td>All-E</td>
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</tr>
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</table>

Note. Full-length Ya cDNA with mutations in all potential MAPK phosphorylation sites to alanine (All-A) or to glutamic acid (All-E) were cloned into pGBT9 vector. The resulting construct was cotransfected with pGAD424 (the negative control), pGAD424-P0, or pGAD424-lamin (the positive control). β-Gal color and liquid assays were performed with each cotransfection.
with preimmune serum did not precipitate either P0 or YA (Fig. 5A, lanes 3 and 5), indicating that the precipitation of YA and P0 is specific to anti-P0 antibodies. The YA and P0 bands were also not from anti-P0 antibodies or the protein A beads used for immunoprecipitation, since immunoprecipitation of buffer alone with anti-P0 antibodies showed neither of the bands (Fig. 5A, lane 1). This indicates that YA and P0 are in the same protein complex in both ovarian and embryo cytoplasmic extracts. The ratio of YA to P0 is similar in immunoprecipitates from ovary and embryo cytoplasmic extracts; however, in the supernatants, the ratio of YA to P0 is much higher in embryo samples than in ovary samples, suggesting that there is more YA unassociated with P0 in embryos than in ovaries. Since P0 is more abundant than YA, we interpret the higher level of P0-free YA in embryos to mean that embryo YA has a lower affinity for P0 than ovary YA, rather than that there is only limited availability of P0 in embryos.

In the converse immunoprecipitation experiment, incubation of cytoplasmic extracts from embryo with affinity-purified anti-YA antibodies also precipitated both YA and P0. As shown in Fig. 5B, anti-YA antibodies precipitated both P0 and YA from wild-type extracts, but it precipitated neither YA nor P0 from cytoplasmic extracts of embryos whose YA was a truncated mutant form that is not recognized by the anti-YA antibodies we used (Lin and Wolfner, 1991; Liu et al., 1995).

We used sucrose density gradient analysis to further confirm that YA and P0 are in the same protein complex in cytoplasmic extracts. Though P0 is distributed in all fractions, its abundance peaks in the same gradient fractions as YA’s in the gradient (highest amount in fraction 8 and slightly less in fraction 9), consistent with the conclusion that YA and P0 are members of a complex. This complex migrates at 20S in the sucrose gradient (Fig. 6). Although P0 is predicted based on its sequence to be a component of the large ribosomal subunit (Grabowski et al., 1991), the 20S complex that contains P0 and YA is not a ribosomal subunit; it is smaller than even the small ribosomal subunit (40S). Moreover, another Drosophila ribosomal component, DS3 (Wilson et al., 1994), did not comigrate with this 20S complex (data not shown). Together with the coimmunoprecipitation experiments, these results strongly suggest that YA and P0 are present in the same protein complex in vivo, in both embryos and ovaries, and that ovarian YA is...
likely to associate with this complex more strongly than does embryo YA.

MAPK Phosphorylates YA in Vitro and This Phosphorylation Increases Binding of YA to P0

The changes in YA’s phosphorylation state at a potential MAPK and p34\textsuperscript{Cdc2} kinase site during egg activation suggested that YA may be phosphorylated by MAPK or p34\textsuperscript{Cdc2} kinase in oocytes and dephosphorylated after the initiation of egg activation (Yu et al., 1999). Therefore, we tested whether YA can be phosphorylated by one or both of these kinases.

Purified MBP-HMK-YA was incubated in vitro under phosphorylation-permissive conditions with p34\textsuperscript{Cdc2} kinase immunoprecipitated from mitotic Hela S3 cells, with MAPK, or with both p34\textsuperscript{Cdc2} kinase and MAPK. As shown in Fig. 7, MBP-HMK-YA was not phosphorylated in the presence of active p34\textsuperscript{Cdc2} kinase alone (Fig. 7, lane 1), but was phosphorylated in the presence of MAPK alone and of both kinases (Fig. 7, lanes 2 and 3). These results indicate that, in vitro, YA is a substrate for MAPK but not for p34\textsuperscript{Cdc2} kinase.

We then tested whether phosphorylation of MBP-HMK-YA by MAPK affects MBP-HMK-YA’s binding of P0. Equal amounts of phosphorylated MBP-HMK-YA and unphosphorylated MBP-HMK-YA (from a “mock” phosphorylation reaction) were incubated with the same amount of purified P0-His\textsubscript{6}. The complex was pulled down with amylose beads. At 0.3 μg, a concentration of MBP-HMK-YA below saturation for binding, more P0 was pulled down together with phosphorylated MBP-HMK-YA (Fig. 8, lane 3) than with unphosphorylated MBP-HMK-YA (Fig. 8, lane 4). This indicates that MAPK phosphorylated YA binds more strongly to P0 than unphosphorylated YA in vitro.

Decreased P0 Levels Partially Suppress a Hypomorphic YA Mutant Allele

For further confirmation of interaction between YA and P0, we examined the genetic interaction between YA and P0. We examined the effect of maternal heterozygosity for a P0 null allele P0\textsuperscript{306} (Frolov and Birchler, 1998) on embryos from females homozygous for the hypomorphic Ya\textsuperscript{77} mutation (“Ya\textsuperscript{77} embryos”). Ya\textsuperscript{77} embryos are classified into three categories based on their arrest phenotype (Liu et al., 1995). Pronuclear arrest embryos are arrested at the pronuclear stage at the end of meiosis. This is the most severe Arrest phenotype.

FIG. 6. YA and P0 are present in a 20S complex in cytoplasm. Cytoplasmic extracts from 0- to 2-h embryos were loaded onto a 5–35% sucrose density gradient. Fractions were analyzed by Western blotting with anti-YA antibodies (YA; top panel) and anti-P0 antibodies (P0; bottom panel). Both YA and P0 peaked at fraction 8 in the density gradient. This fraction is 20S, based on the size markers run at the same time (data not shown). In addition to their comigration in a 20S complex, YA and P0 appear to also be found (separately) in other complexes.

FIG. 7. YA is phosphorylated by MAPK but not by p34\textsuperscript{Cdc2} kinase. Purified MBP-HMK-YA was incubated with p34\textsuperscript{Cdc2} kinase immunoprecipitated from mitotic Hela cell lysate, MAPK, or both kinases in the presence of [γ-\textsuperscript{32}P]ATP. The kinase reactions were analyzed by SDS-PAGE and autoradiography. MBP-HMK-YA was not phosphorylated by p34\textsuperscript{Cdc2} kinase (lane 1). The lack of signal in this lane is not due to inactivity of the kinase, since the parallel positive control reaction showed histone H1 being phosphorylated by p34\textsuperscript{Cdc2} kinase (lane 4). MBP-HMK-YA was phosphorylated by MAPK in the absence or presence of p34\textsuperscript{Cdc2} kinase (lanes 2 and 3). Phosphorylation of MBP-HMK-YA by MAPK is due to phosphorylation of the YA moiety and not of the MBP-HMK moiety, since the 60-kDa MBP-HMK-containing degradation product in the MBP-HMK-YA preparation is not labeled.
phenotype. Type II embryos escape the pronuclear arrest and undergo a few rounds of nuclear division before arresting. Type I embryos are thought to be degenerating from pronuclear or type II arrests. As shown in Table 2, lowering the P0 level by half (P0306/H11001) partially but significantly (P = 0.02) suppressed the Ya77 phenotype. Fewer embryos from X^X, y Ya77 c vvf/Y; P0306/H11001 mothers arrested at the pronuclear stages than those from control X^X, y Ya77 c vvf/Y; /H11001/MKRS mothers, and more embryos escaped the pronuclear arrest and underwent a couple of rounds of division (Type II). This suggests that P0 normally functions to antagonize Ya function, consistent with our proposal that P0 functions to keep Ya out of nuclei, where Ya functions. Heterozygous P0 null females are fertile, suggesting that half the normal dose of P0 is still enough to retain hyperphosphorylated Ya in the cytoplasm of oocytes.

**DISCUSSION**

The subcellular location of Ya proteins is modulated during development; this correlates with changes in Ya’s phosphorylation state, including at one putative MAPK target site (Yu et al., 1999). In this study, we showed that four proteins from cytoplasmic extracts bind to Ya directly in vitro and identified one of them as Drosophila P0/AP3. Our observations here that the cytoplasmic protein P0/AP3 associates with Ya in a 20S complex in the cytoplasm and that this association is stronger when Ya has been phosphorylated by MAPK suggest the following model (Fig. 9): During oogenesis, Ya is synthesized from maternal mRNA and phosphorylated by MAPK in the developing egg chamber. This phosphorylation causes more Ya to be retained in a 20S cytoplasmic complex that serves to retain Ya outside of nuclei. Upon egg activation, Ya is partially dephosphorylated, including at one MAPK site. This dephosphorylation decreases Ya’s retention by the 20S complex. Those Ya molecules released from the complex are freed to enter nuclei via their NLS. The future purification and identification of the other three cytoplasmic proteins that bind to Ya will identify their roles in the retention of Ya during development. Our data from in vitro assays (affinity chromatography, blot overlay, and pull-down) and from yeast two-hybrid analysis are consistent with this model. The coimmunoprecipitation and the sucrose density gradient experiments with cytoplasmic extracts strongly argue that

**TABLE 2**

Lowered P0 Level Partially Suppresses a Hypomorphic Ya Mutant Allele

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Pronuclear arrest</th>
<th>Type II</th>
<th>Type I</th>
</tr>
</thead>
<tbody>
<tr>
<td>X^X, Ya77; P0306/+ (n = 158)</td>
<td>112 (70.9%)</td>
<td>7 (4.4%)</td>
<td>39 (24.7%)</td>
</tr>
<tr>
<td>X^X, Ya77; +/MKRS (n = 105)</td>
<td>90 (85.7%)</td>
<td>2 (1.9%)</td>
<td>13 (12.4%)</td>
</tr>
</tbody>
</table>

Note. Embryos at 0–2 h from X^X, y Ya77 c v f/Y; P0306/+ and X^X, y Ya77 c v f/Y; +/MKRS females were collected and stained with DAPI. Embryos were scored for their arrest phenotype according to Liu et al. (1995). X^X, Ya77 stands for X^X, y Ya77 c v f/Y. \( \chi^2 \), 7.81; degrees of freedom, 2; P = 0.02.
YA and P0 are together in a complex in vivo and also support this model. Our genetic analysis showing the suppression of a weak Ya allele by lowered P0 dosage is also consistent with the model.

This model has several implications for understanding macromolecular events before and during egg activation.

Our data suggest that MAPK is active in meiosis-arrested Drosophila oocytes. MAPK activity is known to increase during oocyte maturation and remain high in meiotic metaphase-arrested oocytes of, for example, Xenopus and mouse (Colas and Guerrier, 1995; Russo et al., 1998), but to our knowledge this has not been demonstrated in Drosophila. Yu et al. (1999) had shown that 443Thr, a potential MAPK and P34CdC2 kinase phosphorylation site in YA, is phosphorylated in oocytes. Our results here show that MAPK but not P34CdC2 kinase can phosphorylate YA in vitro. Taken together, these suggest that MAPK phosphorylates YA in vivo in oocytes, which suggests that MAPK activity will be detected in Drosophila oocytes, though such activity could potentially be restricted in time or place, as is the case for p34CdC2 kinase in early Drosophila embryos (Su et al., 1998). Because YA is not a target for p34CdC2 kinase in vitro, our data cannot address whether MPF activity is high in meiotic metaphase-arrested oocytes as it is, for example, in Xenopus (Gerhart et al., 1984; Masui and Markert, 1971; Smith and Ecker, 1971).

Our data suggest that modulation of MAPK phosphorylation occurs during egg activation in Drosophila. Yu et al. (1999) had shown that dephosphorylation of a potential MAPK and P34CdC2 target site, 443Thr, during egg activation correlated with changes in YA's subcellular location. That YA is, at least in vitro, a target of MAPK but not P34CdC2 kinase suggests that MAPK activity is modulated. It is possible that modulation of MAPK phosphorylation is a general regulatory mechanism during Drosophila egg activation, similar to other organisms. Future studies of other proteins active or activated at this time in Drosophila (Axton et al., 1994; Chen et al., 2000; Edgar et al., 1986; Elfring et al., 1997; Fenger et al., 2000; Freeman and Glover, 1987; Freeman et al., 1986; Lieberfarb et al., 1996; Loppin et al., 2000; Page and Orr-Weaver, 1996; Santamaria, 1983; Santamaria and Gans, 1980; Shamanski and Orr-Weaver, 1991) will be useful in testing this.

YA may provide a link between MAPK activity and regulation of chromosome condensation state. YA is likely to be involved in modulating chromatin condensation during the end of meiosis (Liu et al., 1995). It has been shown in mice that chromosome condensation state follows MAPK, but not MPF, activity during oocyte metaphase arrest and egg activation (Verlhac et al., 1994). If the same holds true for Drosophila, it would be tempting to postulate that MAPK does so via its regulation of proteins that modulate chromosome condensation, such as YA.

The exclusion of YA from nuclei prior to egg activation (Yu et al., 1999), and its chromatin binding (Lopez and Wolfner, 1997) and nuclear organization (Liu et al., 1995) functions, might suggest that exclusion of YA from nuclei might be essential for oogenesis. Perhaps it is important to exclude from meiotic nuclei a protein that is needed for postmeiotic chromatin remodeling, in order not to interfere with meiotic chromosome condensation. If this were the case, then disabling potential phosphorylation sites that control YA's subcellular location during development should result in meiotic arrest. Our finding here that YA is a target of MAPK makes it possible to test this hypothesis.

We identified a 20S cytoplasmic complex that contains YA and P0 and that we propose serves to retain YA in the cytoplasm in response to developmental signals. Modulation of a protein's interaction with a cytoplasmic protein is one of the modes in which phosphorylation regulates a protein's nuclear entry (Jans and Hubner, 1996). The nuclear entry of NF-kB and Dorsal have been shown to be regulated in this fashion (Drier et al., 1999; Li and Sedivy, 1993; Whalen and Steward, 1993). It will be interesting to see whether the 20S cytoplasmic complex that includes YA, or other analogous complexes, regulates the subcellular location of other Drosophila maternal proteins before and during egg activation.

The molecular events leading to and during Drosophila oocyte metaphase arrest and egg activation are not yet well understood. Our identification of an interaction between YA and P0, and its capacity for regulation by MAPK, points to the involvement and regulation of MAPK activity in these critical developmental processes. This regulation of YA's subcellular localization, and hence its ability to access chromatin where it could potentially sense or modulate condensation, could suggest a mechanism for MAPK's role in the regulation of chromosome condensation state at this critical developmental time in Drosophila.

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