Oocyte Activation and Passage through the Metaphase/Anaphase Transition of the Meiotic Cell Cycle Is Blocked in Clams by Inhibitors of HMG-CoA Reductase Activity

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Abstract. Cell cycle progression for postembryonic cells requires the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-R), the enzyme which catalyzes the production of the isoprenoid precursor, mevalonate. In this study, we examine the requirements of HMG-R activity for cell cycle progression during the meiotic and early mitotic divisions using oocytes and dividing embryos from the surf clam, *Spisula solidissima*. Using two different inhibitors of HMG-R, we find that the activity of this enzyme appears to be required at three distinct points of the cell cycle during meiosis. Depending on the stage at which these inhibitors are added to synchronous clam cultures, a reversible cell cycle block is triggered at the time of activation or at metaphase of either meiosis I or II, whereas there is no block to the mitotic cell cycle. Inhibition of HMG-R activity in activated oocytes does not affect the transient activation of $p42^{MAPK}$ but results in a block at metaphase of meiosis I that is accompanied by the stabilization of cyclins A and B and $p34^{cdc2}$ kinase activity. Our results suggest that metabolites from the mevalonate biosynthetic pathway can act to influence the process of activation, as well as the events later in the cell cycle that lead to cyclin proteolysis and the exit from M phase during clam meiosis.

PROGRESS through the cell cycle of postembryonic cells requires the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-R)[1] (for reviews see Goldstein and Brown, 1990; Maltese, 1990). HMG-R is a microsomal enzyme which catalyzes the reduction of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonate, an essential precursor required for the biosynthesis of several vital end products, which include: cholesterol and other sterols, important for membrane replication and structure; dolichol, required for N-linked protein glycosylation; isopentenyladenine, which is present in some types of tRNAs; and the isoprenoid side-chains of ubiquinone and heme-a, which are involved in electron transport of the mitochondrial respiratory chain (for reviews see Goldstein and Brown, 1990; Maltese, 1990). In addition, the mevalonate-derived isoprenoids, farnesol (15 carbon) and geranylgeranol (20 carbon), are moieties which are post-translationally attached to a diverse set of cellular proteins. These proteins that become isoprenylated include the p21 ras protein (Casey et al., 1989; Hancock et al., 1989; Schafer et al., 1989), small ras-like GTP-binding proteins, the γ subunit of trimeric GTP-binding proteins, nuclear lamins, and yeast mating factors (for reviews see Clarke, 1992; Schafer and Rine, 1992). In some instances the attachment of the isoprenyl groups has been found to be important for cellular localization and function.

Treatment of dividing postembryonic cells with inhibitors of HMG-R activity triggers an arrest in the G1 phase of the cell cycle (Cornell et al., 1977; Kaneko et al., 1978; Quesney-Huneeus et al., 1979; Habenicht et al., 1980; Perkins et al., 1982; Fairbanks et al., 1984; Doyle and Kandutsch, 1988; Chakrabarti and Engleman, 1991; Keyomarsi et al., 1991). A similar G1 arrest is triggered as well in a cell line containing an inactivating mutation in HMG-synthetase, the enzyme which produces HMG-CoA, when grown in the absence of mevalonate (Sinensky and Logel, 1985). The mevalonate requirement for G1 traversal for these cell types is a requirement for a non-sterol product(s), since the addition of mevalonate, but not cholesterol, can overcome the

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1. *Abbreviations used in this paper*: CPM, counts-per-minute; CSF, cytostatic factor; GVBD, germinal vesicle breakdown; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HMG-R, HMG-CoA reductase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MTOC, microtubule organizing center; PBST, PBS containing 0.1% Triton X-100; 1-D TLC, one-dimensional thin-layer chromatography; 2-D TLC, Two-dimensional thin-layer chromatography.

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cell cycle arrest. The identity and function of the mevalonate-derived compound(s) necessary for cell cycle progression in these cells is unknown, although a few candidates have been proposed including dolichol (Larsson, 1985, 1987; Kabakoff et al., 1990), isopentenyladenine (Quesney-Huneeus et al., 1980; Faust and Dice, 1991), prenylated proteins (Sinensky and Logel, 1985; Maltese and Sheridan, 1987; Wolda and Glomset, 1988; Sepp-Lorenzino et al., 1991), and mevalonate itself (Cuthbert and Lipsky, 1990, 1991). In addition to serving as positive regulatory influences in cell cycle progression, increased intracellular levels of mevalonate, or its immediately derived metabolites, appear to negatively influence the process of cell division as well (Cuthbert and Lipsky, 1991).

The requirements of HMG-R activity for cell cycle progression in mitotically dividing early embryos may be different than those seen for postembryonic cells. Inhibition of HMG-R activity does not affect the ability of early sea urchin embryos to divide mitotically. The requirements for HMG-R in these embryos are not manifested until the time of gastrulation (Carson and Lennarz, 1979). Whether or not HMG-R activity is required for mitosis in early embryos from other species is not known. In addition, the requirements of HMG-R for progress through the meiotic cell cycle has not been examined in detail in any biological system.

To examine the contribution of HMG-R activity for cell cycle progression during meiosis and the early mitotic divisions, we have used oocytes and early embryos from the surf clam, *Spisula solidissima*. Oocytes from these animals are activated and induced to undergo the two meiotic divisions and the subsequent mitotic divisions with exquisite synchrony after fertilization in vitro. The mitotic cell cycle of early embryos, which is much more simple than that of postembryonic cells, is driven by the periodic activation and inactivation of the $p34^{cdc2}$ protein kinase, which is required for entry into M phase and exit from M phase, respectively (for review see Murray and Kirschner, 1989; Nurse, 1990). The activation and inactivation of the $p34^{cdc2}$ kinase is driven by the periodic accumulation and destruction of the cyclin proteins, which were first discovered in early embryos of sea urchins and clams as a class of proteins, the levels of which change dramatically as a function of the cell cycle (Evans et al., 1983). The cyclins steadily accumulate during interphase of each cell cycle until late M phase, when they are specifically destroyed. The cyclins act to drive the embryonic cell cycle by forming complexes with the $p34^{cdc2}$ kinase, which is a necessary event to initiate its activity and to drive the entry into M phase. At the end of M phase, the cyclins are proteolyzed, an event that is required for the inactivation of the $p34^{cdc2}$ kinase and the exit from M phase. In clams, there are two cyclins, A and B, that have been identified and the manner in which the levels of these proteins change during the meiotic and mitotic cell cycles has been elucidated (Swenson et al., 1986; Westendorf et al., 1989). Both clam cyclins A and B bind to $p34^{cdc2}$, in separate complexes, to initiate its activation (Draetta et al., 1989; Parker et al., 1991; Roy et al., 1991).

The meiotic cell cycle is different than the mitotic, embryonic cell cycle in that, during the specialized meiotic divisions, two rounds of M phase take place without an intervening period of DNA synthesis. As for the mitotic divisions, the entry into M phase of each meiotic division is preceded by the activation of cyclin/$p34^{cdc2}$ kinase complexes, and the exit of this phase is correlated with a preceding loss of activity of these complexes. In some species, additional protein components have been identified, which are functional during meiosis but not mitosis (Sagata et al., 1988), that affect the activation and inactivation of cyclin/$p34^{cdc2}$ complexes.

A very early event that occurs in clam oocytes, after fertilization, is the activation of a 42-kD mitogen-activated protein kinase ($p42^{MAPK}$; Shibuya et al., 1992). This enzyme is activated as a consequence of phosphorylation on a tyrosine, as well as a threonine residue (for review see Cobb et al., 1991; Thomas, 1992). In fertilized or parthenogenetically activated clam oocytes, $p42^{MAPK}$ becomes tyrosine phosphorylated and activated as a transient, one-time early event, and then is inactivated by the time of meiosis I (Shibuya et al., 1992).

In this study, two different inhibitors of HMG-R were used to examine the requirements of this enzyme activity at the time of clam oocyte activation and during the subsequent meiotic and mitotic cell cycles. We find that HMG-R activity appears to be required at three distinct points of the cell cycle during meiosis, but not mitosis. The effects of these inhibitors on the transient activation of $p42^{MAPK}$ and on the changing levels of the cyclins and $p34^{cdc2}$ kinase activity during the meiotic cell cycle are examined as well.

Materials and Methods

Clam Cultures and Sample Collection

Spisula solidissima oocytes and sperm were collected and fertilization was carried out as described by Westendorf et al. (1989). Oocytes were suspended at a concentration of 40,000/ml in filtered sea water and cultured at 18°C. To parthenogenetically activate, KCl (40 mM) was added to oocyte cultures. In most cases, cells from these cultures, after germinal vesicle breakdown (GVBD), were pelleted and suspended in an equal volume of fresh, filtered sea water. Progress through the cell cycle was monitored by lacto-orcein staining of fixed cells (Kuriyama et al., 1986; Westendorf et al., 1989; Hunt et al., 1992), which were visualized by bright-field microscopy. Culture samples to be used for immunoblotting or kinase assays were collected into ice-cold calcium-free sea water, centrifuged briefly in a microfuge, and the cell pellet was isolated and flash-frozen in liquid N_2. Samples to be used for immunoblotting were thawed by dissolving in hot sample buffer (Laemmli, 1970) to a final cell concentration of ~500 cells/μl. Samples (10 μl) were loaded for SDS-PAGE followed by immunoblotting, as described below. Frozen samples, collected for kinase assays, were homogenized in T buffer (300 mM glycine, 120 mM K-gluconate, 100 mM taurine, 100 mM Hepes, 40 mM NaCl, 15 mM $MgCl_2$, 20 mM EGTA; adjusted to pH to 7.3 with KOH) containing 0.5% NP-40, 10 mM NaF, 40 mM β-glycerophosphate, 1 mM Na-vanadate, chymostatin (2.5 μg/ml), leupeptin (2.5 μg/ml), antipain (2.5 μg/ml), pepstatin A (2.5 μg/ml), and PMSF (15 μg/ml), to a final cell concentration of ~200,000 cells/ml, by passage twenty times through a 26.5-g needle attached to a 1-cc syringe. Homogenates were cleared by centrifugation at top-speed (16,000 *g*) in a microfuge for 5 min at 4°C and the supernatants collected on ice.

Immune/Affinity Precipitations and Kinase Assays

Extracts were assayed for histone H1 or MBP phosphorylating activities directly by incubating 10 μl (10 μg of protein) of the cleared supernatant, prepared as described above, with 10 μl of kinase buffer containing 40 mM Hepes-NaOH (pH 7.3), 10 mM EGTA, 20 mM $MgCl_2$, 0.2 mg/ml myelin basic protein (Sigma Chem. Co., St. Louis, MO) or 0.2 mg/ml histone H1 (Boehringer Mannheim Biochemicals, Indianapolis, IN), 10 μM protein kinase A peptide inhibitor (Sigma Chemical Co.), 0.5 mCi/ml [γ-^{32}P]ATP (3,000 Ci/mmol) and 200 μM ATP (diluted 1:100 from a 2.0-mM stock prepared in 10 mM Tris-HCl, pH 7.3, and stored at −80°C) for 10 min at 22°C. For the H1 kinase assays, the reactions were terminated by the addition of

80 μl sample buffer and were boiled for 5 min. These samples were electrophoresed on 13% gels, which were dried and used for autoradiography. For the MBP phosphorylation assays, the reactions were terminated by placing 15 μl of the sample onto p81 Whatman filters, which were immediately placed into 1% phosphoric acid. Filters were washed three times in 1% phosphoric acid, once in acetone, dried, placed into scintillation fluid, and the cpm were measured. For immune precipitation/kinase assays, 150 μl (150 μg of protein) of the cleared homogenate, prepared as described above, was first precleared with 50 μl of a 1:1 bead-to-buffer slurry of protein A-Sepharose beads (Sigma Chemical Co.) for 1 h at 4°C with rotation. Beads were pelleted by a brief microfuge spin, the supernatant removed, antisera (2 μl) was added, and samples were rotated overnight at 4°C. Protein A-Sepharose beads (50 μl of a 1:1 slurry) were added, incubated similarly for 1 h, and the beads were then washed three times with cold buffer (0.5 M LiCl, 0.1 M Tris-HCl, pH 7.2) and two times with cold buffer containing 20 mM Tris-HCl, pH 7.2, 100 mM NaCl. Beads were then pelleted, the supernatant removed, and kinase assay buffer containing 7.5 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.2, 1 mM $ZnCl_2$, 1 mg/ml histone H1, 0.8 μM ATP, and 0.6 mCi/ml [γ-^{32}P]ATP (3,000 Ci/mmol) was added to the pellets. Samples were incubated for 10 min at 22°C, at which time, the reaction was terminated by the addition of 60 μl sample buffer and a 5-min boil. Samples were prepared for autoradiography after SDS-PAGE, as described above. p13^{suc1} protein was bacterially expressed, purified, and coupled to CNBr-Sepharose CL-4B as described by Brizuela et al. (1987). For affinity precipitation, 75 μl of cleared homogenate was mixed with 30 μl p13^{suc1} beads, after preclearing with protein A-Sepharose beads, as described for the immune precipitations, and incubated for 1 h at 4°C. Bead precipitates were washed, assayed for kinase activity, and analyzed by SDS-PAGE and autoradiography, as described for the immune precipitations.

Immunoblot Analysis and Antibodies

Affinity-purified anti-cyclin A and anti-cyclin B antibodies were produced and characterized as described by Swenson et al. (1986) and Westendorf et al. (1989), respectively. Anti-phosphotyrosine antibodies (clone 4G10) were obtained from U.B.I. (Lake Placid, NY). Cell pellets, dissolved in sample buffer, prepared as described above, were separated by SDS-PAGE, blotted onto nitrocellulose (Towbin et al., 1979), incubated with antibodies, and then with alkaline-phosphatase conjugated secondary antibodies (Promega Biotec., Madison, WI), and developed according to the manufacturer's instructions.

Inhibitors

Lovastatin and simvastatin were kindly provided by Dr. Alfred Alberts of Merck Sharp & Dohme Research Laboratories (Rahway, NJ). PD123588 was a generous gift of Dr. Roger Newton of Parke-Davis Pharmaceutical Research (Ann Arbor, MI). Inhibitors (10 mM in DMSO) were added to clam cultures, with stirring, to final concentrations of 35 μM (lovastatin), 20 μM (simvastatin), or 50 μM (PD123588) Emetine (Sigma Chemical Co.) was stored at −20°C as a 20-mM stock solution in water and was added to clam cultures to a final concentration of 100 μM.

[^{14}C]Acetate Labeling/Analysis

Oocytes were suspended in Hepes-buffered artificial sea water (380 mM NaCl, 50 mM $MgCl_2$, 30 mM Na_2SO_4, 10 mM KCl, 10 mM $CaCl_2$, 100 mM Hepes-NaOH, pH 8.0) at a concentration of 57,000 cells/ml and incubated with [^{14}C]acetate (3 μCi/ml; 110 mCi/mmol; Moravek Biochemical, Inc., Brea, CA). At the end of the labeling period, 4 ml of culture samples were collected and washed with an excess of ice-cold calcium-free sea water, centrifuged briefly and the cell pellet was isolated and flash-frozen in liquid N_2 until analysis. Samples were thawed by vortexing sequentially in 2 ml methanol, 1 ml chloroform, and 0.7 ml H_2O. An additional 1 ml chloroform and 1 ml H_2O was added and samples were centrifuged at 1,600 *g* for 5 min, to create aqueous and organic phases (Bligh and Dyer, 1959). 3 ml of each aqueous phase was removed, lyophilized, resuspended in 100 μl H_2O, and a portion of this fraction (25 μl) was analyzed by two-dimensional TLC (2-D TLC) on cellulose plates developed with 1-propanol/NH_3 (25% aqueous solution)/H_2O (6:3:1) in the first dimension and 2-butanol/acetic acid/H_2O (40:10:13) in the second dimension (Beyer et al., 1985). Standards chromatographed in parallel were mevalonate, farnesyl pyrophosphate, geranylgeranylpyrophosphate, HMG CoA, acetyl CoA, acetoacetyl CoA, [^{14}C]acetate, and isopentenylpyrophosphate. Chromatograms of labeled compounds were subjected to autoradiography and unlabeled standards were identified by iodine visualization. Radiolabeled chromatographic spots, which were subjected to further analysis, were scraped and eluted from the gel matrix with H_2O. A portion of eluted samples were subjected to base treatment (0.5 N NaOH, 60°C, 1 h), neutralized with HCl, lyophilized, and suspended in H_2O for 2-D TLC analysis. Counts-per-minute in radiolabeled chromatographic spots were determined by scintillation counting after scraping and resuspension of gel matrix in scintillant.

Immunofluorescence

At the time of GVBD, activated clam oocytes were pelleted and suspended in an equal volume of calcium-free sea water and further incubated. Samples were collected and settled onto polylysine-coated coverslips for 5 min before submerging into 2% 1,3 dichloroacetone/acetone, which was in a dry ice/methanol bath, for 10 min. Coverslips were rehydrated by successive, 3-min incubations in: 90, 70, 50, and 25% acetone before placement in PBS. After washing two times (5 min) in PBS containing 0.1% Triton X-100 (PBST), coverslips were incubated in blocking buffer (2% goat serum in PBST) for 30 min at room temperature. After a 5-min wash in PBST, samples were incubated with anti-α-tubulin antibody (Amersham Corp., Arlington Heights, IL) diluted 1:100 at 4°C overnight. After washing four times for 10 min in PBST (the third wash also contained 2% goat sera), samples were incubated with rhodamine-labeled secondary antibodies (Boehringer Mannheim Biochemicals) at 1:100 dilution for 2 h at room temperature. After further washes (two times in PBST, one time in PBST containing 1 μg/ml HOECHST's 33342 [Calbiochem, San Diego, CA], two times in PBS, each for 10 min), coverslips were mounted in a solution containing 0.2 M Tris-HCl, pH 8.5, 20% Mowiol (Calbiochem, San Diego, CA), and 2.5% 1,4 diazoabicyclo[2,2,2]octane (Sigma Chem. Co.). Samples were observed using a laser scanning confocal microscope (model 600; Bio-Rad Laboratories, Hercules, CA).

Results

Inhibitors of HMG-CoA Reductase Activity Block Activation of Clam Oocytes

The female gamete of the surf clam is an oocyte that is arrested at prophase of meiosis I with condensed chromosomes distributed within a large germinal vesicle (nucleus). Upon fertilization, the oocyte is activated, i.e., the cell cycle block is removed, and meiosis is rapidly resumed. At 10–15 min after fertilization, GVBD occurs synchronously and the chromosomes begin congressing to form the metaphase plate for meiosis I. Meioses I and II take place in close succession and the embryos then proceed directly into the mitotic divisions (for the timing of the meiotic cell cycle stages, see Fig. 5, *top panel*). Activation of clam oocytes can also be triggered parthenogenetically by treatment with KCl (Allen, 1953). For these oocytes, GVBD and the two meiotic divisions occur with the same timing as seen for those activated by fertilization. Artificially activated oocytes, however, are unable to undergo the mitotic divisions due to the absence of centrioles, which are normally acquired from the sperm at fertilization and which are required for the formation of the bipolar spindles during mitosis (Kuriyama et al., 1986).

Although the requirements for HMG-R activity for cell cycle progression in postembryonic cells have been well documented, the contribution of this activity in oocytes for initiation of the cell cycle, or in early embryos for passage through the meiotic and mitotic divisions have not been examined in detail. We first examined the effects of inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-R) on clam oocyte activation. Oocytes were incubated in the presence of the HMG-R inhibitor, lovastatin, for 15 min, and

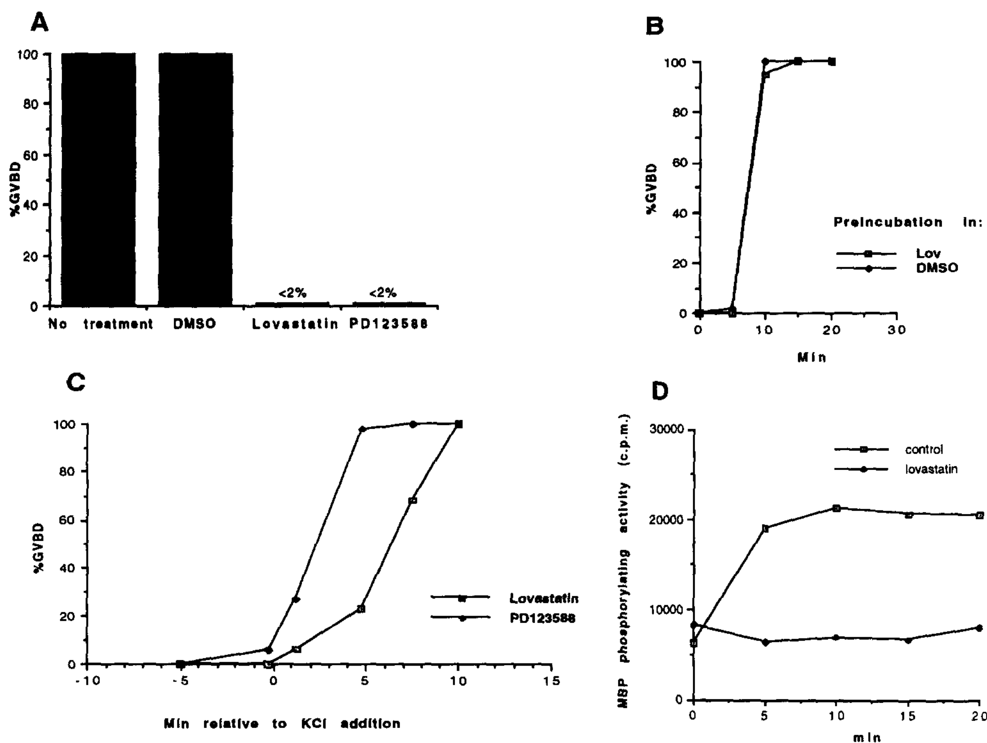

Figure 1. Effects of HMG-R inhibitors on oocyte and MAP kinase activation. (*A*) Clam oocytes were first incubated for 15 min in lovastatin, PD123588, an equal volume of vehicle alone (DMSO) or untreated sea water, and then treated with KCl to activate. At 20 min and 60 min after KCl addition, oocytes were examined by bright-field microscopy and the percentage of those which had undergone GVBD were determined. Between 50–100 oocytes were scored for each time point. In those cultures which were untreated (No treatment) or treated with vehicle alone (DMSO), 100% GVBD had occurred by 20 min, whereas less than 2% GVBD had occurred in cultures treated with either lovastatin or PD123588 at 20 min or 60 min. (*B*) Clam oocytes were incubated for 15 min in either lovastatin or equal volumes of DMSO. Oocytes were pelleted, washed, and starting at 5 min after the first wash, were incubated in fresh sea water to which KCl was added (time 0′) to activate. At successive 5′ intervals, samples were collected into fixative and the chromosomes stained with lacto-orcein. Samples were examined by bright-field microscopy and the percentage of oocytes which had undergone GVBD was determined. Between 50–100 oocytes were scored for each time point. (*C*) Oocytes were treated with lovastatin or PD123588 at times before and after KCl treatment and the percentage of GVBD was determined at 60 min after addition of KCl by bright-field microscopy. (*D*) Oocytes were treated with lovastatin or equal volumes of DMSO for 15 min before the addition of KCl. At the time of KCl addition, and at subsequent 5-min intervals, samples were collected and flash frozen. Samples were thawed by homogenization in buffer and assayed for MBP phosphorylating activity. The resulting filter-bound cpm were determined for each sample.

then treated with activating concentrations of KCl. The occurrence of GVBD in these oocytes was monitored by bright-field microscopy as an assay of activation. In control, untreated cultures or those treated with vehicle alone, GVBD had occurred in 100% of the culture by 20 min after KCl addition (Fig. 1 *A*). However, in cultures incubated with lovastatin, less than 2% GVBD had occurred by 60 min after KCl addition. The lovastatin-induced block to GVBD was reversible. When oocytes were preincubated with lovastatin, washed in fresh sea water and treated with KCl, GVBD occurred with the same timing as control cultures (Fig. 1 *B*). These results demonstrate that lovastatin can reversibly block clam oocyte activation and, therefore, suggest that HMG-R activity is required for this process.

Reversal of the lovastatin-induced arrest of KCl-treated oocytes by mevalonate, the product of HMG-R would unequivocally establish the requirement of HMG-R activity for clam oocyte activation. However, in experiments in which lovastatin-arrested clam cultures were incubated in the presence of [^{3}H]mevalonate, we were unable to detect cellular incorporation of ^{3}H (not shown). These results indicated that this agent was not taken up effectively by these cells, thus depriving this study of a desired control which would demonstrate the specificity of the lovastatin effects. The addition to lovastatin-arrested clam oocytes of downstream components in the mevalonate biosynthetic pathway, cholesterol, dolichol, and coenzyme Q, which are hydrophobic compounds, like lovastatin itself, also were unable to abrogate the arrest in the presence of KCl (data not shown). These results, however, are difficult to interpret in the absence of proof that these agents were actually being taken up.

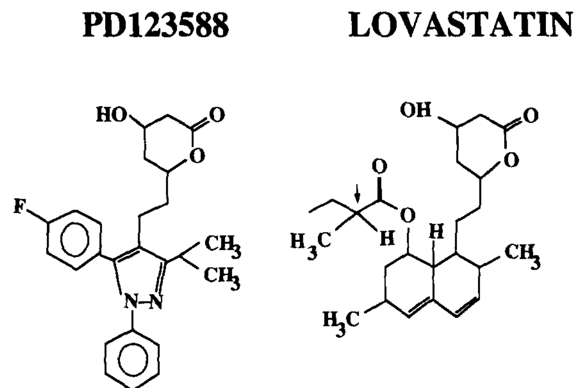

Figure 2. Inhibitors of HMG-R. The structures of the two HMG-R inhibitors mainly used in this study are shown. The structure of simvastatin, a third inhibitor used, is identical to that of lovastatin, except for the presence of a methyl group which displaces the hydrogen at the position shown by the arrow.

A second inhibitor of HMG-R, simvastatin, was tested for effects on clam oocyte activation. The effects of this inhibitor were identical to those seen with lovastatin; oocyte activation was blocked reversibly (not shown). Simvastatin is a derivative of lovastatin and, as such, its structure is almost identical, except for a single methyl group which is substituted in the side chain ester (Fig. 2). It was possible that the block to oocyte activation induced by these inhibitors was due to effects specific to these types of compounds on unknown molecular targets rather than to inhibition of HMG-R activity.

To test whether or not the HMG-R inhibitor-induced block to activation of clam oocytes was due to the inhibition of HMG-R activity or to effects specific to lovastatin-like compounds on unknown targets, we examined the effects on oocyte activation with a different type of HMG-R inhibitor. For these experiments, we used PD123588, a compound that is structurally dissimilar to lovastatin (Fig. 2) and is slightly less active than both lovastatin and simvastatin in its effectiveness of inhibiting HMG-R in vitro (Alberts et al., 1980; Endo, 1980; Sliskovic et al., 1990; Endo, 1992). As was the case for lovastatin, PD123588 acted to block clam oocyte activation (Fig. 1 *A*). The PD123588-induced arrest was also readily reversed when oocytes were washed and incubated in the presence of fresh sea water (not shown). These results corroborate the notion that HMG-R activity is required for activation of clam oocytes and, thus, suggest that a metabolite(s) from the mevalonate biosynthetic pathway can act to influence this process.

The time window in which the HMG-R inhibitors could act as effective blockers of activation was determined relative to the time of KCl treatment of oocyte cultures. For these experiments, lovastatin or PD123588 was added to cultures at specific times before and after KCl addition and the percentage of GVBD in these cultures was determined at 5-min intervals. Fig.1 *C* shows the results obtained by 60 min after KCl treatment for cultures treated with lovastatin or PD123588. Lovastatin was able to block greater than 50% GVBD if added any time up to 6 min after KCl treatment. It was completely ineffective at blocking activation if added after 10 min. PD123588 was effective at blocking GVBD up to 3 min after KCl treatment and was ineffective after 5 min. This difference in the time windows of oocyte sensitivity to these inhibitors may be due to a difference in the efficiency of their uptake by the cells. In these experiments, untreated control cultures assayed in parallel underwent GVBD synchronously between 10–15 min after KCl treatment (not shown). These data suggest that HMG-CoA reductase activity is required for at least 3–6 min after KCl treatment in order for oocytes to become activated.

An early event after fertilization or KCl treatment of clam oocytes is the activation of a myelin basic protein (MBP)-phosphorylating activity (Shibuya et al., 1992). This activity appears by 5 min after fertilization or KCl activation, before GVBD, and is due, at least in part, to the combined activities of p42 mitogen-activated kinase and $p34^{cdc2}$/cyclin B complexes (Shibuya et al., 1992). The effects of HMG-R inhibitors on activation of this MBP-phosphorylating activity after KCl treatment were tested. Oocyte cultures were incubated for 15 min in lovastatin or vehicle alone, and then treated with activating concentrations of KCl. Samples were collected at successive 5-min intervals from both cultures and used to prepare extracts, which were assayed for MBP-phosphorylating activity (Fig. 1 *D*). In samples from control cultures, MBP-phosphorylating activity was stimulated by 5 min after KCl addition. This activity was not stimulated in the lovastatin-treated culture. These results indicate that lovastatin acts to block this early event of activation, as well as GVBD, and suggests that HMG-R activity is required for this process.

Inhibition of HMG-R in Oocytes Leads to a Build-Up of HMG-CoA

To directly examine the components in the mevalonate biosynthetic pathway that are synthesized in oocytes and the effects on the synthesis of these components conferred by HMG-R inhibition, clam oocytes were treated with lovastatin or vehicle alone (DMSO) and incubated for 60 min with [^{14}C]acetate, a small molecule that is taken up by oocytes. Samples were extracted to produce an organic fraction, expected to contain the sterols and dolichol, and an aqueous fraction, expected to contain acetyl CoA, acetoacetyl CoA, HMG-CoA, mevalonate, and other non-sterol mevalonate derived metabolites, as well as metabolites from the citric acid cycle (Bligh and Dyer, 1959). A sample containing the non-saponifiable simple lipids was prepared from the organic fraction (Dittmer and Wells, 1969) and analyzed by 1-D TLC with dolichol, Coenzyme Q and the sterols, cholesterol, squalene, and lanosterol standards analyzed in parallel. No labeled oocyte components comigrated with these standards (data not shown) suggesting that these components are not synthesized in high levels by oocytes. These results were consistent with our observations that the addition of cholesterol, dolichol, or Coenzyme Q did not abrogate the block to activation in lovastatin-arrested oocytes.

Equivalent amounts of aqueous fractions from oocyte cultures treated with lovastatin or DMSO alone and biosynthetically labeled with [^{14}C]acetate were analyzed by 2-D TLC and autoradiography (Fig. 3, *A* and *B*). There are a number of radiolabeled chromatographic spots, which appear to be identical, that were detectable in these fractions from both cultures (Fig. 3, spots *4–10*). We have not identified these components, but suspect them to include metabolites from the citric acid cycle. Radiolabeled components that cochromatographed with mevalonate, isopentenyl pyrophosphate, farnesyl pyrophosphate, or geranylgeranylpyrophosphate standards were not detected in either of these samples. The transient presence of these intermediates as they are produced during passage through the mevalonate biosynthetic pathway may have rendered their detection below the limits of sensitivity in this analysis.

Several labeled chromatographic spots were detected in the lovastatin-treated oocytes, which were not detected in those oocytes treated with DMSO (Fig. 3 *B*; spots *1–3*). Spots *2* and *3* did not chromatograph with any of our standards and remain unidentified. Spot *1*, however, chromatographed in the same position as the HMG-CoA standard. To confirm that the radiolabeled component from spot *1* was HMG-CoA, this spot was scraped, eluted, and treated with base to disrupt the thiol ester linkage of HMG-CoA. When this sample was analyzed by 2-D TLC, a single radiolabeled component was generated which chromatographed in the same position as a radiolabeled spot from a ^{14}C-HMG-CoA standard treated with base and analyzed in parallel (not

shown). These results indicated that the radiolabeled component represented in spot *1* was HMG-CoA.

To compare the synthesis of HMG-CoA, and other radiolabeled components from aqueous fractions, at successive times, oocytes were treated with lovastatin or DMSO and incubated continuously with [^{14}C]acetate. Samples were removed at 20 and 40 min for extraction, the aqueous fractions were prepared and equivalent amounts were analyzed by 2-D TLC and autoradiography (data not shown). Spots *1–10* were scraped and the counts-per-minute (CPMs) determined by scintillation counting. When samples from the lovastatin and DMSO-treated cultures were compared, the levels of radioactivity in spots *2* and *4–10* either were maintained at roughly unchanged levels, or increased with time in similar ways from the two cultures (data not shown). The CPMs from spots *1* (HMG-CoA) and *3* increased dramatically over time in the lovastatin-treated culture samples but not from those treated with DMSO. The CPMs from the HMG-CoA spots from these cultures are shown in Fig. 3 *C*. These results indicate that oocyte treatment with the HMG-R inhibitor, lovastatin, leads to the build up of HMG-CoA as well as a second component, and, thus, confirm that HMG-R is being inhibited by lovastatin in these cells. The build-up of these components in cells treated with the HMG-R inhibitors might be important in the effects on cell cycle progression that are observed.

HMG-R Inhibitors Induce an Arrest at Metaphase of the Meiotic Cell Cycle of Activated Clam Oocytes

To examine the effects of inhibiting HMG-CoA reductase on cell cycle progression at times after activation, clam oocyte cultures were fertilized or treated with activating concentrations of KCl, allowed to undergo GVBD and, at successive times, aliquots of these cultures were removed and incubated in the presence of lovastatin. Samples from each aliquot were removed at 5-min intervals and put into fixative for chromosome staining and cell cycle staging. Results from such an experiment in which oocytes were activated by KCl treatment are shown in Figs. 4 and 5.

In the untreated control culture, chromosomes were clearly visible by 5 min after activation as tightly condensed bodies distributed within the germinal vesicle (Fig. 4 *A*). By 10 min, the germinal vesicle had broken down and the chromosomes had begun to congress. Congression continued until ~30 min, with the formation of the metaphase plate (an en face view of the metaphase plate is shown in Fig. 4 *A*, 30 min). Anaphase of meiosis I occurred at about this same time, as shown, giving rise to the first polar body. A polar body, which is slightly out of the plane of focus, can be seen in Fig. 4 *A* at 50 min. The chromosomes remaining in these oocytes immediately realigned on the second meiotic metaphase plate and anaphase of meiosis II occurred at about 50 min as shown.

A quantitative representation of cell cycle progression for the control culture is shown in Fig. 5, *top panel*, in which the percentage of cells undergoing GVBD, anaphase I, anaphase II, and those with condensed chromosomes are plotted as a function of time. The chromosomes remained condensed until anaphase II was completed, whereupon they underwent a round of decondensation and recondensation. In fertilized control cell cultures, this period of chromosome decondensation represented the time of the first round of DNA synthesis before mitosis 1. KCl-activated cultures did not undergo mitosis. The chromosomes from these cultures, however, asynchronously underwent a round of decondensation and recondensation starting at ~100 min, as

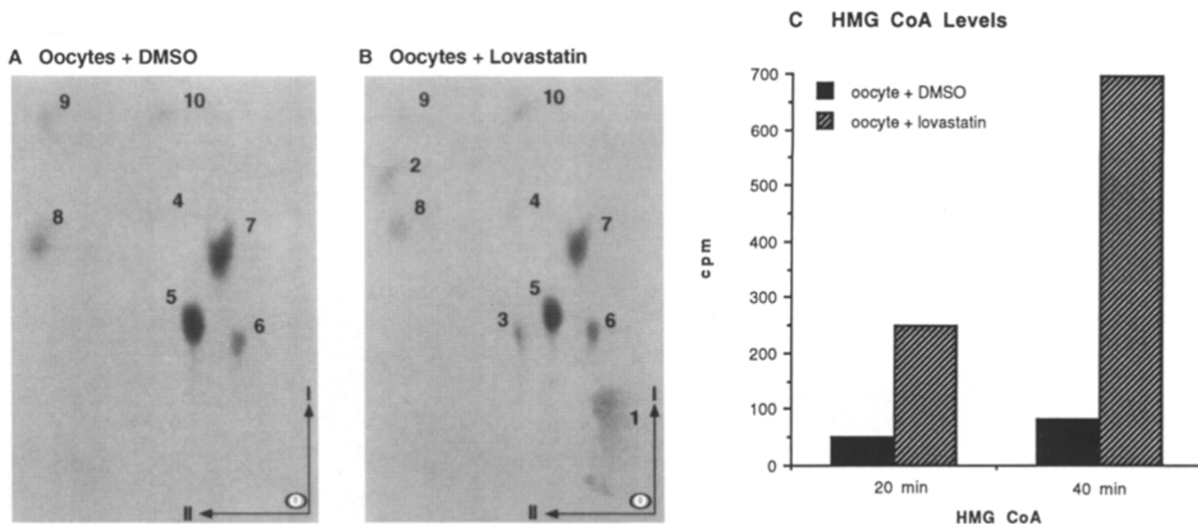

Figure 3. HMG-R inhibitor treatment of clam oocytes results in increased levels of HMG CoA. (*A* and *B*) Oocytes were treated with either DMSO (0.6%) or lovastatin (50 μM) and incubated with [^{14}C]acetate (3 μCi/ml; 110 mCi/mmol) for 60 min. Cells were washed, extracted into an organic and aqueous fractions and equal portions of the aqueous fractions were applied to cellulose plates (*O*, origin). Samples were separated by two-dimensional thin-layer chromatography and exposed for autoradiography. The metabolite represented by spot *1* was found to be HMG CoA. *I* and *II* indicate the first and second dimensions, respectively. (*C*) Oocytes were treated with either DMSO or lovastatin and incubated with [^{14}C]acetate, as described for *A* and *B*. At successive times (20 and 40 min), samples were removed from the culture, oocytes were washed, extracted and equal portions of the aqueous fractions were separated by 2-D TLC and exposed for autoradiography as described for *A* and *B*. HMG CoA spots were scraped and the counts-per-minute were measured and plotted as shown.

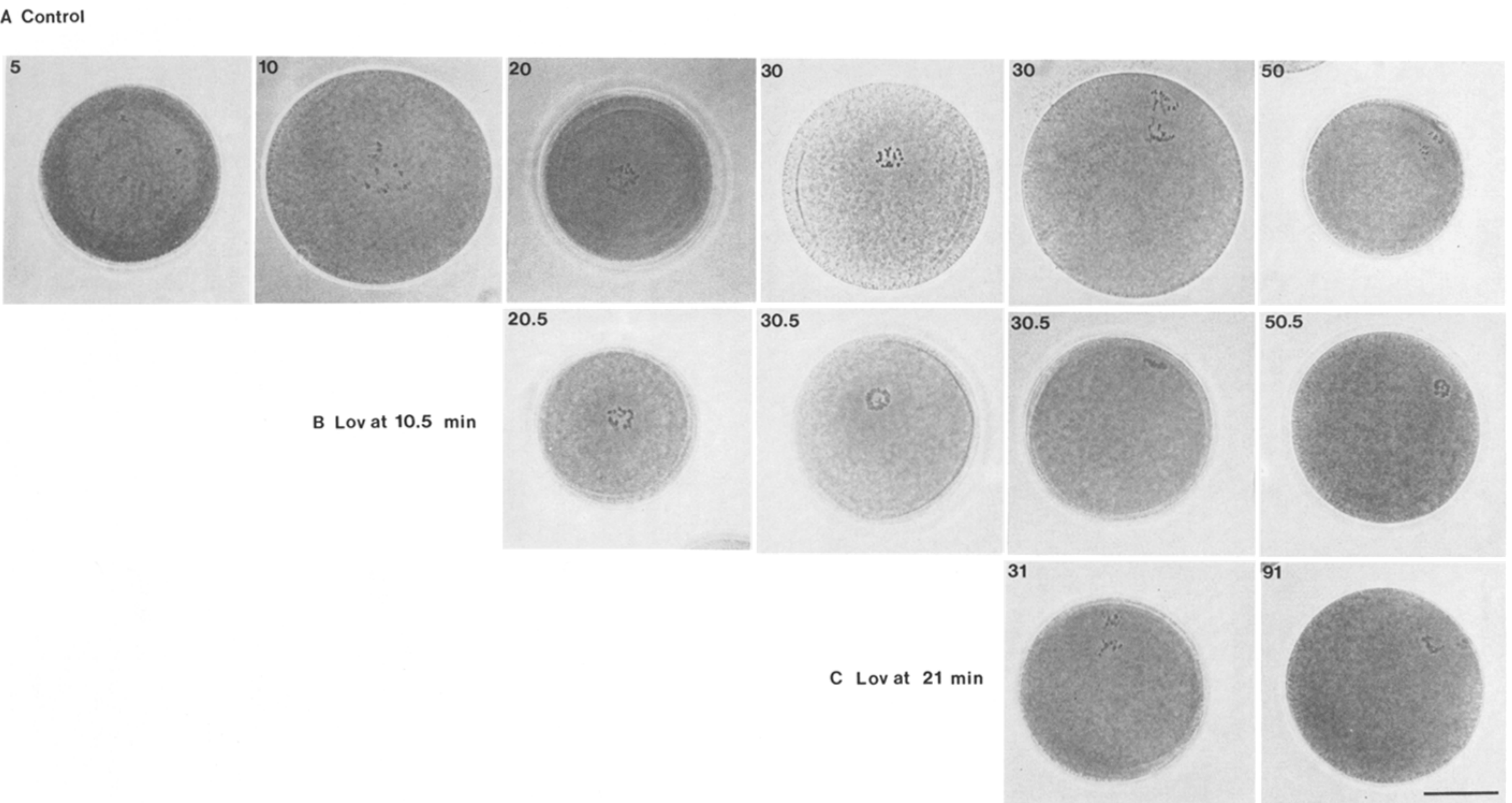

Figure 4. HMG-R inhibitors block activated clam oocytes at metaphase of meiosis. Clam oocytes were synchronously induced to undergo meiosis by artificially activating with KCl and, at successive times, aliquots of the culture were removed and incubated in the continuous presence of lovastatin. Samples from each culture were collected at successive 5-min intervals and put into fixative. The chromosomes of these fixed cells were stained with orcein and visualized by bright-field microscopy. Shown are oocytes at selected times from the control culture (*A*), and from the cultures to which lovastatin was added at 10.5 min (*B*) and 21 min (*C*) after KCl addition. The time of fixation, after KCl addition, for samples shown in each field is indicated. Oocytes incubated with lovastatin starting at 10.5 min arrest at metaphase of meiosis I and those incubated with lovastatin starting at 21 min arrest at metaphase of meiosis II. Bar length is 20 μM.

shown for the experiment represented in the top panel of Fig. 5.

The chromosomes from cells which were removed from the control KCl-activated culture and incubated with lovastatin starting at 10.5 min continued to congress and to form a metaphase plate by ~ 30 min, in a manner similar to controls (Fig. 4 *B* and Fig. 5, *second panel*). At this point, however, these cells became arrested. The metaphase plate in these cells was maintained out to the last collection point, which was 90 min (Fig. 5, *second panel*). A metaphase plate of meiosis I from this culture is shown at 50.5 min, a time when control cultures were undergoing anaphase of meiosis II (Fig. 4 *B*). When the 90-min metaphase-arrested cells from this culture were washed and diluted into fresh sea water, they went on to form polar bodies (not shown) indicating that this lovastatin-induced arrest was reversible. A reversible arrest at metaphase of meiosis I was also induced in embryos from fertilized cultures treated similarly (see Fig. 6).

Cells that were removed from control cultures and incubated with lovastatin starting at 21 min were not inhibited from undergoing anaphase of meiosis I. The timing and synchrony of anaphase in these cultures was similar to that of controls (Fig. 5, compare third panel with first panel). Anaphase chromosomes from a cell in such a culture at 30 min is shown in Fig. 4 *C*. After anaphase I, the chromosomes in these cells realigned on the metaphase plate for meiosis II, similar to those cells from the control culture. At this point in the cell cycle, however, they became arrested and maintained this metaphase plate for the duration of the experiment (Fig. 5, *third panel*). A metaphase plate for meiosis II, as well as the polar body from first meiosis (slightly out of the plane of focus) in a cell from this culture at 91 min can be seen in Fig. 4 *C*. The metaphase arrest in these cultures was also readily reversible when cells were washed and placed in fresh sea water (not shown). These same results were obtained from oocyte cultures activated by fertilization, as well (not shown).

When aliquots from the control culture were removed and incubated with lovastatin at 31.5 min, there was no effect on the ability of the cells to undergo meiosis II (Fig. 5, *fourth panel*). As can be seen in Fig. 5, there were some minor effects on the timing of anaphase II for this culture, which was slightly delayed relative to controls (50% of cells in the culture had undergone anaphase II by 56 min compared to 48 min of controls), and on the synchrony of division, which was slightly lessened. The chromosomes in this culture underwent a round of decondensation and recondensation after meiosis II, although the timing and synchrony of these events were also slightly affected relative to those in control cultures. At the last collection point (110 min), the chromosomes were still condensed (Fig. 5, *fourth panel*). In fertilized cell cultures treated similarly, the embryos went on to divide mitotically, although more slowly than embryos from control cultures and in an asynchronous manner (not shown).

The addition of lovastatin to an aliquot of the control culture at 42 min after activation had no effect on the completion of anaphase II or the subsequent process of chromosome decondensation and recondensation, which occurred in a manner similar to the control culture (Fig. 5, *last panel*). The chromosomes at 115 min were still condensed in this culture. Embryos from fertilized cultures treated similarly divided mitotically. However, again, the rate of division was reduced and the synchrony appeared to be lost (not shown).

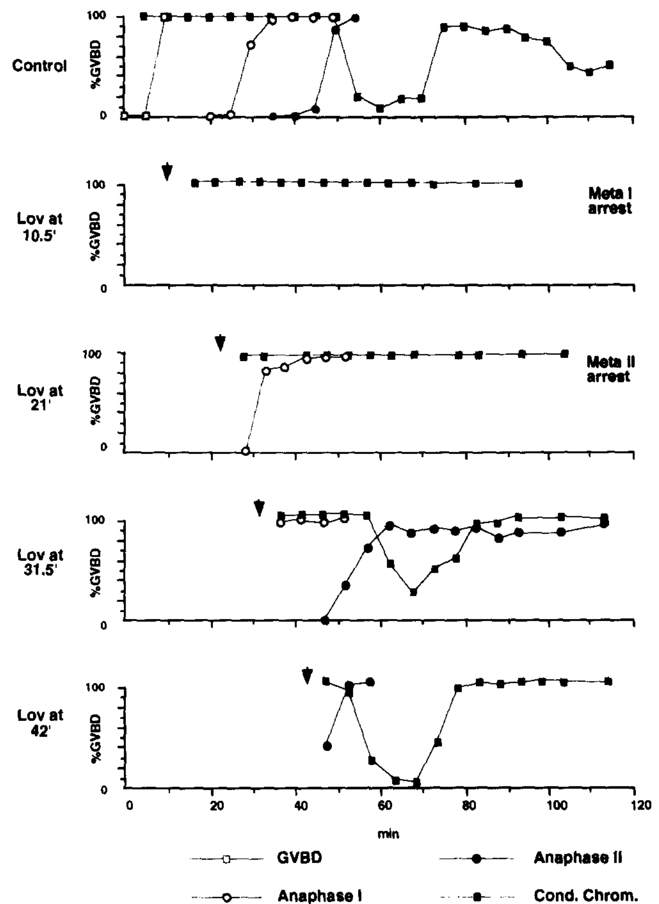

Figure 5. Cell cycle staging of activated oocytes treated with HMG-R inhibitors. The cell cycle stage for culture samples collected in the experiment described in the legend for Fig. 3 was determined quantitatively. Samples collected from control cultures (*top panel*), and those incubated with lovastatin starting at 10.5 min (*second panel*), 21 min (*third panel*), 31.5 min (*fourth panel*), and 42 min (*bottom panel*) after KCl addition were examined by bright-field microscopy and the percent of cells in each which had undergone: (*1*) germinal vesicle breakdown (*GVBD*; *open squares*); (*2*) anaphase of meiosis I (*Anaphase I*; *filled diamonds*); (*3*) anaphase of meiosis II (*Anaphase II*; *filled circles*); and (*4*) those in which the chromosomes were condensed (*Cond. Chrom*; *filled squares*) were determined. Between 50–100 oocytes were examined for each time point. Large arrowheads indicate the times at which lovastatin was added for each culture.

These experiments were repeated using PD123588 to inhibit HMG-R instead of lovastatin and the same results were obtained (not shown). We have not examined in a quantitative way the effects of these inhibitors on mitotic cell cycle progression, except to note that incubation of embryos with concentrations of these inhibitors that block meiotic anaphase do not prevent mitosis from occurring, although it appears to occur more slowly than in untreated cultures and in an asynchronous manner.

These results demonstrate that two different inhibitors of HMG-R induce a reversible block at metaphase during meiosis in activated clam oocytes and embryos, indicating that HMG-R activity is required for meiotic cell cycle progression. The activity of this enzyme appears to be required dur-

ing separate time windows for the successful enactment of anaphase for meiosis I and II, respectively; active HMG-R is required sometime between 10.5 and 21 min postactivation for anaphase I and sometime between 21 and 31.5 min for anaphase II. These results suggest that a metabolite(s) from the mevalonate biosynthetic pathway can act at these times to influence the passage from metaphase to anaphase during the meiotic cell cycle in the clam.

Inhibitors of HMG-R Stabilize Cyclin Levels and p34cdc2 Kinase Activity

Chromosome dynamics during the cell cycle is driven by changing levels in the activities of cyclin/p34cdc2 complexes. The activities of these complexes, at a basic level, is driven by the changing levels of the cyclin proteins. We tested whether the meiotic metaphase arrest induced by inhibitors of HMG-R was due to an effect on cyclin/p34cdc2 activities and cyclin levels, or was due to a more direct effect on chromosome dynamics. For these experiments, the levels of the two clam cyclins, A and B, and the levels of histone H1 kinase activity, which is a measure of p34cdc2 kinase activity, was determined in control, untreated cultures and those treated with HMG-R inhibitors. In clam embryos, new protein synthesis is not required for activation or the proper timing or passage through meiosis I. It is required, however, for the proper enactment of meiosis II (Hunt et al., 1992). To rule out potential negative effects of the HMG-R inhibitors on protein synthesis, we focused our efforts on analyzing the effects of these inhibitors during meiosis I.

Oocytes were activated by KCl treatment or fertilization, allowed to undergo GVBD, and then an aliquot of the culture was removed and treated with one of the HMG-R inhibitors to induce an arrest at metaphase I. At successive times, samples each were collected from untreated cultures and inhibitor-treated cultures and processed for cell cycle staging, immunoblots to determine the levels of cyclins A or B, or for assays of H1 kinase activity.

Shown in Fig. 6 are the results of an experiment carried out using a fertilized culture which was treated with lovastatin. The immunoblots of samples reacted with anti-cyclin A antibodies are shown in *A*. As we have seen in earlier work (Swenson et al., 1986), cyclin A was not detectable in oocytes. In control samples it appeared at ~20 min after fertilization and accumulated until 30 min, the time of anaphase I, when the levels dropped. The levels of this protein reaccumulated until anaphase II (~50 min), dropped again, and then continued to increase at successive times to 75 min, which was the last time point of sample collection. In samples treated with lovastatin starting at 12 min after fertilization, cyclin A was detectable starting at 22 min. The levels slightly increased by 27 min, and then were maintained throughout the course of the experiment. The periodic decline in the levels of this protein, due to specific degradation that occurred in the control culture was not seen in the lovastatin-treated culture which became arrested at metaphase I.

Immunoblots of the same samples analyzed in *A* were reacted with anti-cyclin B antibodies (Fig. 6 *B*). Cyclin B was detected in oocytes of the control cultures (Westendorf et al., 1989). The levels of this protein did not change appreciably at anaphase I (30 min), increased until anaphase II (50 min), at which time there was a marked drop, and then

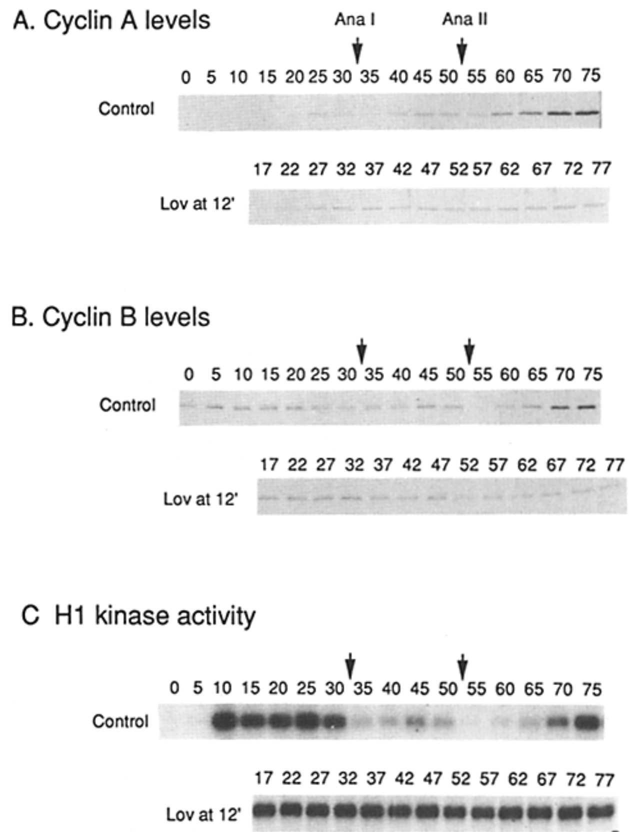

Figure 6. Metaphase I arrest induced by HMG-R inhibitors is accompanied by stabilized levels of the cyclins and histone H1 kinase activity. Clam oocytes were fertilized and, at 12 min after fertilization, half of the culture was removed and incubated in the continued presence of lovastatin. At 5-min intervals three separate samples were removed from each culture. One sample was put into fix for chromosome staining and the other two samples were collected into ice cold calcium-free sea water, to halt cell cycle progression, washed and flash-frozen. One set of samples was thawed directly into hot SDS gel electrophoresis sample buffer and boiled for 5 min. The relative levels of cyclins A and B in these samples were determined, after SDS-PAGE of equal amounts of protein and immunoblotting by incubation with either anti-cyclin A (*A*) or anti-cyclin B (*B*) antibodies. The remaining set of samples was thawed by homogenization in buffer and assayed for histone H1 kinase activity. The products of this reaction (^{32}P-labeled histone H1) were separated by SDS-PAGE and detected by autoradiography (*C*). At the top of each gel lane shown in *A*, *B*, and *C* is the time of collection for that particular sample. Cell cycle progression for both cultures was quantitated by examining chromosome-stained samples at each time point as described in the legend for Fig. 4. The time at which 50% of the embryos in the control culture had undergone anaphase of meiosis I (*Ana I*; 34 min) or of meiosis II (*Ana II*; 53 min) is indicated by the arrowheads in each panel. The culture treated with lovastatin did not undergo anaphase but became arrested at metaphase of meiosis I.

reaccumulated over time until the 75-min time point. In samples from the lovastatin-treated culture, the levels of cyclin B appeared to remain unchanged from 17 min throughout 77 min, indicating that cyclin B, as for cyclin A, was stabilized by inhibition of HMG-R.

H1 kinase activity was low in oocytes of the control culture, but increased dramatically by 10 min, the time of GVBD (Fig. 6 *C*). This activity remained high until 30 min

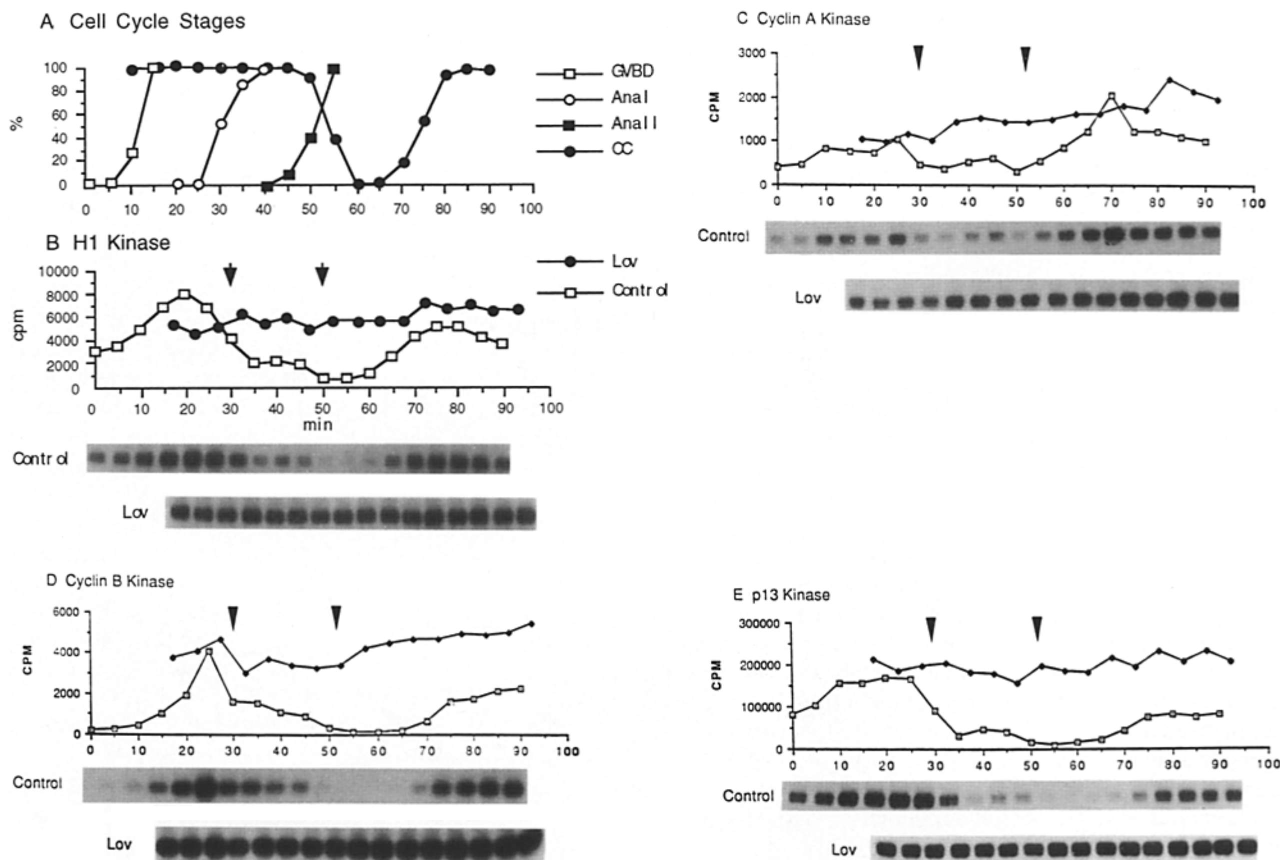

Figure 7. $p34^{cdc2}$ kinase activity in immune precipitates of cyclins A or B, or in affinity precipitates with p13 beads is stabilized at high levels in clam embryos arrested at metaphase I arrest by HMG-R inhibitors. Clam oocytes were activated with KCl and, at 14 min after KCl addition, an aliquot of the culture was removed and incubated in the presence of lovastatin. Samples from both control and lovastatin-treated cultures were collected at successive 5-min intervals, as for the experiment described in the legend for Fig. 5. The chromosomes of fixed samples were stained and the cell cycle stage of each sample was determined quantitatively as described in the legend for Fig. 4. These data for the control culture are shown in *A*. The lovastatin-treated culture became arrested at metaphase I. Extracts from a set of frozen samples, which were thawed by homogenization in buffer, were split into several aliquots and were assayed for histone H1 kinase activity either directly (*B*), or after immune precipitation with anti-cyclin A antibodies (*C*) or anti-cyclin B antibodies (*D*), or after affinity precipitation with $p13^{suc1}$ beads (*E*). The products of these reactions (^{32}P-labeled histone H1) were separated by SDS-PAGE and detected by autoradiography (*B–E*). The gel bands giving rise to the autoradiograph shown in *B–E* were cut out, the counts-per-minute (CPM) measured and plotted, as shown in the top part of each of these panels. The time at which 50% of the embryos in the control culture had undergone anaphase of meiosis I (*Ana I*; 30 min) or of meiosis II (*Ana II*; 52 min) is indicated by the arrowheads in each panel.

(anaphase I) when it dropped precipitously. The activity subsequently increased to a slightly higher level to 50 min, when it again declined sharply, and then reaccumulated in samples collected until 75 min. In the lovastatin-treated culture, H1 kinase activity was high at 17 min and was maintained at a high level throughout the course of the experiment (Fig. 6 *C*).

When these experiments were repeated using PD123588 to inhibit HMG-R instead of lovastatin, the same results were obtained (not shown). Similar results were also obtained when oocyte cultures were activated by KCl treatment rather than by fertilization (see Fig. 7).

H1 kinase assays provide a measure of the total $p34^{cdc2}$ kinase activity present in cellular extracts. We examined whether or not the kinase activity of subpopulations of $p34^{cdc2}$ present in complexes with either cyclin A or cyclin B, or those which could associate with $p13^{suc1}$ affinity beads, was differentially affected by the presence of HMG-R inhibitors. $p13^{suc1}$ is a protein that binds $p34^{cdc2}$ and $p34^{cdc2}$-like kinases (Brizuela et al., 1987; Dunphy et al., 1988). It also appears to bind, at least partially, the $p42^{MAPK}$ from activated clam embryos (Shibuya et al., 1992). This kinase, however, does not phosphorylate histone H1, which was used as a substrate in our kinase assays. These experiments were carried out in a similar method to that described above, except that oocytes were activated by KCl treatment rather than by fertilization. Samples were again collected for cell cycle staging and for H1 kinase assay. In addition, samples were also collected and prepared for immune precipitation with antibodies for either cyclins A or B, or for affinity-precipitation with $p13^{suc1}$ beads. These precipitates were then assayed for kinase activity using histone H1 as an exogenous substrate (Fig. 7).

A quantitative representation of cell cycle progression is shown for the control culture in Fig. 7 *A*. The times when 50% of the embryos had undergone meiotic anaphase I or II were ~30 min and 52 min, respectively. Fig. 7 *B* shows the data from the total H1 kinase assays of these samples.

These results were similar to those obtained for the experiment represented in Fig. 6. Total H1 kinase activity in these samples changed over time in control cultures in a manner similar to that seen before (compare Fig. 7 *B* to Fig. 6 *C*) and was stabilized at high levels in the lovastatin-treated culture, which became arrested at metaphase I.

In samples from control cultures, total H1 kinase activity, as well as that associated with each type of precipitation, changed over time in similar ways (Fig. 7, *B–E*). In each case, kinase activity was low in oocytes before fertilization, increased to high levels until anaphase I when there was a decline. After this time, the activity for H1 kinase, cyclin A precipitates and $p13^{suc1}$ precipitates increased slightly until anaphase II, when the levels dropped again, and then reaccumulated to higher levels, until 90 min (Fig. 7, *B*, *C*, and *E*). The levels of activity associated with cyclin B declined at anaphase I and did not appear to reaccumulate before anaphase II, when it diminished before increasing again until 90 min (Fig. 7 *D*).

In samples treated with lovastatin, kinase activity remained high, increasing slightly over time until 90 min (Fig. 7, *B–E*). There was a slight decrease in the levels of cyclin B associated kinase activity at 32 min (Fig. 7 *D*). The decline in this activity was not due to a decrease in the levels of cyclin B protein. Immunoblots of cyclin B in lovastatin-treated cultures showed that the levels of this protein were unchanged at this time (Fig. 6 and data not shown).

These results demonstrate that inhibitors of HMG-R, added to clam cultures at a time when they induce a metaphase I arrest, lead to a stabilization of the levels of cyclins A and B as well as a stabilization of high levels of $p34^{cdc2}$ kinase activity found in complexes with cyclins A or B, or that which associates with $p13^{suc1}$ beads. This indicates that the activity of HMG-R is required during meiosis somewhere upstream of the biochemical processes that lead to cyclin destruction and loss of $p34^{cdc2}$ kinase activity normally seen at anaphase, and suggest that a metabolite(s) from the mevalonate biosynthetic pathway can act to influence the proper enactment of these processes.

Protein Synthesis Is Not Required for Maintenance of the Metaphase I Arrest Induced by Inhibitors of HMG-R

The stabilization of the levels of cyclins A and B in embryos arrested at meiotic metaphase by the HMG-R inhibitors, leading, presumably, to the stabilization of $p34^{cdc2}$ kinase activity in these cells, could arise from either a total block in the activation of the cyclin destruction system, or from a partial block. In the latter case, it would be expected that the stabilized levels of the cyclins in these embryos would be due to continued cyclin synthesis. To test whether the HMG-inhibitors were affecting a complete or partial block of the activation of the cyclin destruction system, we examined the effects of inhibiting protein synthesis on the maintenance of metaphase I and stabilization of histone H1 kinase activity in meiotic embryos arrested with lovastatin.

An oocyte culture was incubated with emetine at a concentration that has been shown to inhibit protein synthesis (Hunt et al., 1992), and then activated using KCl. At 13 min after KCl treatment, an aliquot of the culture was removed and incubated with lovastatin. Samples were taken at successive 5-min intervals from both cultures for cell cycle staging, H1 kinase assays, and immunoblots.

The culture treated with emetine alone underwent GVBD at 13 min (not shown) and anaphase I at about 38 min (Fig. 8). The proper enactment of anaphase II did not occur in this culture, an effect of emetine on clam embryos that has been noted by others (Hunt et al., 1992). H1 kinase activity was low in oocytes, increased dramatically around the time of GVBD, and then declined around the time of anaphase I, as seen in activated cultures in the absence of emetine (compare Fig. 8 to Fig. 6 *C*). This activity disappeared after anaphase I and was not detectable in samples collected to 75 min (Fig. 8). In immunoblots of samples from these cultures, cyclin B was present at constant levels up until anaphase I, when the levels declined, and then disappeared, whereas cyclin A was undetectable, confirming that protein synthesis was inhibited (not shown).

The culture incubated with lovastatin, in addition to emetine, became arrested at metaphase I, with condensed chromosomes tightly aligned on the metaphase plate (not shown). This arrest was maintained for the duration of the experiment, until 77 min. H1 kinase activity in this culture was maintained at a high level, from 17 min to 77 min (Fig. 8). Cyclin B was detected at constant levels on immunoblots of samples from this culture, whereas cyclin A was not detected in any of these samples (not shown).

These data demonstrate that maintenance of the metaphase I arrest induced by inhibiting HMG-R activity, does not require protein synthesis. In the absence of protein synthesis, the levels of H1 kinase activity and cyclin B are maintained. These results indicate that the cyclin destruction system is blocked to activation by inhibiting HMG-R and suggest, again, that metabolite(s) from the mevalonate biosynthetic pathway can act somewhere upstream to influence the initiation of this process.

Inhibition of HMG-CoA Reductase Activity Does Not Affect Tyrosine Dephosphorylation of $p42^{MAPK}$

In fertilized or KCl-treated clam oocytes, $p42^{MAPK}$ becomes tyrosine phosphorylated and activated as a transient, one-time event. The activity and tyrosine phosphorylation of this enzyme disappears by the time of meiosis I (Shibuya et al., 1992). We tested whether or not the tyrosine dephosphorylation of $p42^{MAPK}$ seen in untreated clam cultures was affected by inhibiting HMG-R activity. For these experiments, clam oocytes were activated by KCl treatment and at 13 min, after GVBD, an aliquot of the culture was removed and incubated in the presence of lovastatin to induce a metaphase I arrest. Samples were collected at successive 5-min intervals from both cultures for cell cycle staging and immunoblotting using anti-phosphotyrosine antibodies.

In the control, untreated culture, tyrosine phosphorylation of $p42^{MAPK}$ was undetectable in the oocyte, appeared at 5 min, reached a peak at 15 min, and then declined to undetectable levels by 30 min (Fig. 9). After this time, the tyrosine phosphorylation of $p42^{MAPK}$ was not seen in samples from this culture collected until 75 min. Anaphases I and II took place in this culture at ~33 min and 53 min, respectively, as indicated (Fig. 9). In the culture treated with lovastatin, which became arrested at metaphase I, tyrosine phosphorylation of $p42^{MAPK}$ was detected at 17 min at levels

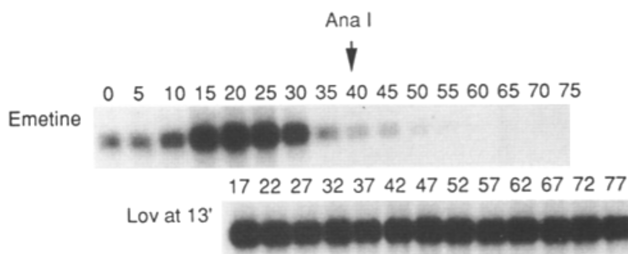

Figure 8. Protein synthesis is not required for maintenance of the metaphase I arrest or stabilization of H1 kinase activity induced by lovastatin treatment. Clam oocytes were incubated for 15 min in the presence of emetine, and then activated by KCl addition. At 13 min after KCl addition, an aliquot of the culture was removed and incubated in the presence of lovastatin. Samples from both cultures were collected at successive 5-min intervals and prepared for cell cycle staging and H1 kinase assays. The time of anaphase I for the culture treated with emetine alone is indicated. Cells from the culture treated with emetine and lovastatin became stably arrested at metaphase I. The products of the H1 kinase assays (^{32}P-labeled histone H1) were separated by SDS-PAGE and detected by autoradiography, as shown.

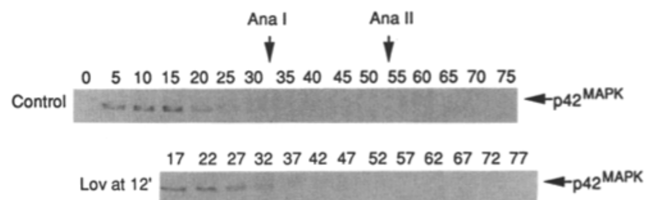

Figure 9. Lovastatin treatment of activated clam oocytes does not affect tyrosine dephosphorylation of p42 MAP kinase. Clam oocytes were activated by KCl treatment and, at 13 min after KCl addition, an aliquot of the culture was removed and incubated in the presence of lovastatin. Samples were collected at successive 5-min intervals and prepared for cell cycle staging and immunoblot analysis using anti-phosphotyrosine antibodies. Shown is a region of the immunoblot which contains the $p42^{MAPK}$ protein as indicated. The time of anaphase I and II for the control, untreated culture is indicated. The lovastatin-treated culture became stably arrested at metaphase I.

similar to that seen in the control culture (Fig. 9). The levels of this modification were maintained until ~27 min, when they declined, disappearing by ~37 min, and were not detected again in samples collected throughout 77 min.

Although the timing of tyrosine dephosphorylation of $p42^{MAPK}$ was slowed for clam cultures incubated with lovastatin, these data demonstrated that the removal of this modification was not prevented by inhibiting HMG-R activity. These results indicate that the maintenance of activity and tyrosine phosphorylation of $p42^{MAPK}$ is not required for the maintenance of metaphase I arrest induced by lovastatin treatment.

Effects of HMG-CoA Reductase Inhibition on Microtubule Polymerization and Spindle Formation

For most types of postembryonic cells, treatments with agents that cause microtubule depolymerization, such as nocodazole, induce a metaphase arrest accompanied by high levels of $p34^{cdc2}$ kinase activity and condensed chromosomes, similar to the arrest that we have obtained with meiotic clam cells treated with inhibitors of HMG-R activity. Although not true for some species of embryonic cells, these same effects of microtubule depolymerizing agents are produced in clam embryos undergoing either meiosis or mitosis (Hunt et al., 1992; and K. Swenson, unpublished). We tested whether or not the metaphase arrest in clams induced by HMG-R inhibitors was potentially due to effects on microtubule polymerization.

For these experiments, clam oocytes were activated with KCl, treated with lovastatin at 12 min to induce a metaphase I arrest, and then at 50 min were fixed, incubated with antitubulin antibodies, and prepared for indirect immunofluorescence detection of microtubules by confocal microscopy. In these samples, polymerized microtubules present in spindle structures were detected (Fig. 10, *A* and *B*). These spindles, however, instead of containing the usual two poles found normally, were unusual in that they contained four poles. Fig. 10 *A* contains the images obtained from three different optical sections of a single cell within which the four poles can be visualized. Fig.10 *B* contains an image from a single optical section of a cell taken through the metaphase plate, which is on its side. Polymerized microtubules emanating from three of the four spindle poles can be seen in this section. The darkened area in the center of these poles are the condensed metaphase chromosomes. The fourth pole is out of the plane of the optical section shown.

These results show that microtubule polymerization and spindle formation is not prevented in activated clam embryos treated with lovastatin. This indicates that the metaphase I arrest induced by inhibiting HMG-R activity is not due to the inability of these cells to polymerize their microtubules at a gross level. The spindles of activated clam oocytes treated with lovastatin and fixed at 50 min contained four poles instead of two. This suggests that inhibiting HMG-R activity during clam meiosis does not interrupt the splitting of the poles, which occurs normally in untreated, control samples before this time (Kuriyama et al., 1986). Normal splitting of the spindle poles in these samples, in the absence of anaphase and cytokinesis, would be expected to give rise to a spindle containing four poles as is seen.

Discussion

Inhibition of HMG-R Blocks Cell Cycle Progression at Three Points during Meiosis in Clams and Stabilizes the Levels of Cyclins and $p34^{cdc2}$ Kinase Activity

This work demonstrates that inhibitors of HMG-R act to induce a cell cycle arrest at three distinct points during meiosis of clam embryos. When added to clam oocyte cultures before fertilization, or until 3–6 min after KCl treatment, HMG-R inhibitors block activation reversibly. If these agents are added to synchronous cultures at times after activation, the meiotic cell cycle continues up to the time of metaphase when an arrest occurs accompanied by the maintenance of condensed chromosomes aligned on the metaphase plate. Reversible cell cycle arrest occurs at metaphase of meiosis I or II depending upon the time in the cell cycle at which these HMG-R inhibitors are added. The addition

A

B

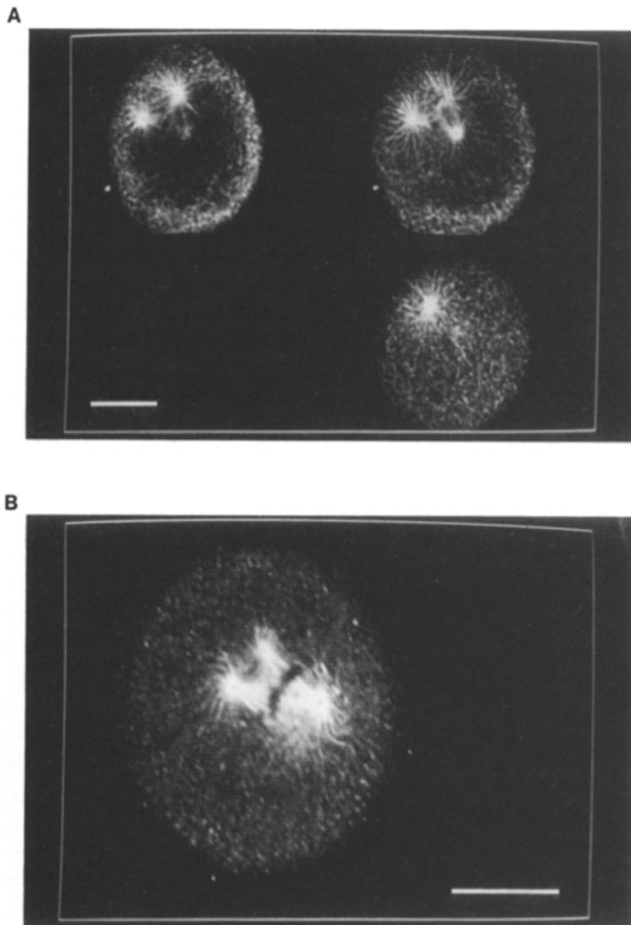

Figure 10. Lovastatin treatment of activated clam oocytes does not affect microtubule polymerization, spindle formation or spindle pole splitting. Clam oocytes were activated by KCl treatment and, at 12 min after KCl addition, were incubated in the presence of lovastatin to induce a metaphase I arrest. At 50 min, oocytes were fixed and prepared for indirect immunofluorescence staining using anti-tubulin antibodies. Samples were mounted for fluorescence viewing by confocal microscopy. (*A*) Three different optical sections (5 μm each) of a single cell in which the four poles of the spindle can be visualized. (*B*) A 5-μm optical section that slices through the metaphase plate, which is on its side, is shown. Three of the four spindle poles present can be seen. Bar length is 20 μM.

of the HMG-R inhibitors to clam cultures, after the time windows of sensitivity, has no effect on the ability of these cells to complete meiosis or to undergo the subsequent mitotic divisions, indicating that these agents are not toxic and that their effects on the cell cycle in this biological system appear to be meiosis-specific. The cell cycle effects of these inhibitors in clams are unique. No other agents, including those which inhibit macromolecular synthesis, have been identified which trigger these effects.

In clam embryos, to which HMG-R inhibitors have been added at a time to induce a metaphase I arrest, the levels of cyclins A, B, and $p34^{cdc2}$ kinase activity do not change over time, but are stabilized at levels that are similar to those found in untreated cultures at the time of metaphase I. Total $p34^{cdc2}$ kinase activity, assayed directly in extracts, or activity from subpopulations of $p34^{cdc2}$, assayed in immune precipitates with cyclins A or B, or in $p13^{suc1}$ precipitates, is maintained at high levels in cells arrested at metaphase I by treatment with HMG-R inhibitors. The work of several groups indicates that the inactivation of $p34^{cdc2}$, which occurs normally at the end of M phase, is an important event for the exit from M phase and requires cyclin destruction. In these experiments, truncated, non-degradable forms of cyclins A or B were introduced in intact cells or in cell-cycle extracts, and shown to prevent the inactivation of $p34^{cdc2}$ kinase activity, chromosome decondensation, spindle disassembly, and cytokinesis (Murray et al., 1989; Ghiara et al., 1991; Luca et al., 1991; Gallant and Nigg, 1992; Verde et al., 1992). It is likely that the stabilization of $p34^{cdc2}$ kinase activity and the metaphase arrest that is induced in activated clam embryos treated with HMG-R inhibitors is due to the effects of these agents in stabilizing the cyclin proteins. Stabilization of the cyclins in these embryos indicates that the activity of HMG-R is required somewhere upstream for the enactment of the normal cellular processes which lead to cyclin proteolysis.

The Maintenance of Metaphase I Arrest Induced by HMG-R Inhibition Does Not Require Protein Synthesis Nor the Maintenance of Activated p42 MAP Kinase

A natural arrest at metaphase of meiosis I occurs normally in the development of female gametes for several species (Zampetti et al., 1973; Guerrier et al., 1986; Dube and Dufresne, 1990). Maintenance of this metaphase I arrest, in oocytes from these species, appears to be dependent upon continued protein synthesis (Zampetti et al., 1973; Neant and Guerrier, 1988; Dube and Dufresne, 1990). In the case of the mollusk, *Patella vulgata*, this requirement for protein synthesis in maintenance of the metaphase I arrest appears to be a requirement for cyclin synthesis since microinjection of anti-sense oligonucleotides complementary to cyclin mRNAs releases this cell cycle block (van Loon et al., 1991).

In contrast, the metaphase I arrest that is triggered in clam embryos by treatment with lovastatin does not require continued protein synthesis. Lovastatin-treated clam embryos, in which protein synthesis is inhibited, arrest at metaphase of meiosis I and maintain stable levels of cyclin B and $p34^{cdc2}$ kinase activity. The presence of cyclin A is not required for the maintenance of the metaphase I arrest, since it is absent from these embryos. The maintenance of cyclin B levels in these embryos, in the absence of protein synthesis, indicates that its continued synthesis is not required for the maintenance of the metaphase I arrest and that a complete block to those processes which lead to cyclin proteolysis is induced by the inhibition of HMG-R activity.

For many vertebrate species, the natural arrest point of the female gamete is at metaphase of meiosis II. This arrest is due to the cytoplasmic activity, cytostatic factor (CSF), first identified in *Xenopus* eggs (Masui and Markert, 1971) and is accompanied by stabilized levels of the cyclin proteins and the activities of $p34^{cdc2}$ kinase and MAP kinase (Ferrell et al., 1991; Gotoh et al., 1991*a*,*b*; Posada et al., 1991; Zaitsevskaya and Cooper, 1992). The CSF-induced metaphase arrest appears to be mediated through its activation and maintenance of MAP kinase activity (Haccard et al., 1993).

In activated clam embryos, $p42^{MAPK}$ is transiently activated, as a one-time event, before meiosis I (Shibuya et al.,

1992). The transient activation of this kinase is not affected in embryos treated with lovastatin at times which induce a downstream arrest at metaphase I. In these cells, then, in contrast to what may be required for a CSF-type of arrest, the activation and maintenance of p42^{MAPK} activity is not required for the maintenance of metaphase arrest, nor for the stabilization of high levels of p34^{cdc2} kinase activity.

Mechanisms of Action for HMG-R Inhibitors

Two types of HMG-R inhibitors mainly are used in this study, lovastatin and PD123588. Both lovastatin, which is isolated as a fungal metabolite (Alberts et al., 1980; Endo, 1980) and PD123588, which is synthetically derived (compound 10 in [Sliskovic et al., 1990]), were developed independently for use as drugs effective in lowering plasma cholesterol in hypercholesterolemic patients. They act to inhibit HMG-R, a microsomal enzyme that catalyzes the reduction of HMG-CoA to mevalonate, which is the rate-limiting step in the synthesis of cholesterol (Goldstein and Brown, 1977). In independent tests, the effectiveness of these compounds to inhibit HMG-R has been carried out and compared to that of compactin, another HMG-R inhibitor related to lovastatin. Lovastatin was found to be slightly more effective than compactin in inhibiting HMG-R (Alberts et al., 1980; Endo, 1980), whereas PD123588 was found to be equally effective (Sliskovic et al., 1990).

The structural commonalities found in all HMG-R inhibitors that have been developed lie in the presence of the upper part of their structures, represented by lactone ring of lovastatin and PD123588 (Fig. 2). The active form of these inhibitors is produced by intracellular esterases that open this ring to create a lactate, which resembles the hydroxymethylglutarate portion of HMG-CoA, the natural substrate of HMG-R. These compounds competitively inhibit the binding of HMG-CoA to HMG-R, apparently by the binding of the lactate portion of the inhibitors to the hydroxymethylglutarate-binding domain in the active site of the enzyme (Nakamura and Abeles, 1985). There is evidence to suggest that the hydrophobic "lower portion" of these inhibitors, which varies widely in structure, binds to a hydrophobic region of HMG-R located near, but not within, the active site (Nakamura and Abeles, 1985). Although effects of these inhibitors on molecular targets other than HMG-R have not been seen, were they to occur, it is likely that these effects would be mediated by the hydrophobic lower portions.

Lovastatin and PD123588, both very effective inhibitors of HMG-R (Alberts et al., 1980; Endo, 1980; Sliskovic et al., 1990), are different in the lower, hydrophobic portion of their structures (Fig. 2). Because of this, we would expect that possible effects they might have on cellular targets, other than HMG-R, would be different. The effects of these agents on the cell cycle of meiotically dividing clams, however, are identical. Thus, we think it highly likely that the effects of these agents in this biological system are due to their actions as HMG-R inhibitors.

In clam oocytes treated with lovastatin, there is a build-up of HMG-CoA, as well as a second unidentified component. This suggests that the effects of the HMG-R inhibitors on activation, as well as on passage through meiotic metaphase, may be due either to a negative influence on these processes by a metabolite that lies upstream of mevalonate, the levels of which accumulate in the presence of these inhibitors, or to the lack of a mevalonate-derived metabolite that is required in a positive way for the enactment of these processes. Although negative effects on cell cycle progression of HMG CoA or other upstream metabolites have not been noted before, the increased intracellular levels of other metabolites in the mevalonate biosynthetic pathway have been seen to negatively influence the process of cell division in other cell types (Cuthbert and Lipsky, 1991). The use of agents that specifically block steps in this pathway upstream of HMG-R, as well as the development of electroporation methods for supplying HMG-R inhibitor-treated clam oocytes and embryos with mevalonate and mevalonate-derived compounds may shed light on this issue.

Requirements of HMG-R Activity for Activation

An early event of clam oocyte activation is the activation of an MBP-phosphorylating activity, which is due to the combined activities of activated p42^{MAPK} and p34^{cdc2} (Shibuya et al., 1992). This activity appears in oocytes within five minutes after fertilization or KCl treatment, which is before the time of GVBD that occurs at ~10 min. We looked directly for the activation of this MBP-phosphorylating activity in oocytes treated with KCl in the presence of HMG-R inhibitors and found none, indicating that the requirement of HMG-R activity is upstream of this process.

The requirement for HMG-R for oocyte activation and GVBD may be species specific. GVBD in *Xenopus* oocytes, after treatment with progesterone, the natural hormone that triggers activation of these cells, is not affected by prior microinjection of compactin, indicating that HMG-R activity is not required for this process (Schafer et al., 1989). However, there are indications that mevalonate-derived compounds may be involved in particular signaling pathways that lead to GVBD in these oocytes. GVBD and meiosis can be triggered in *Xenopus* oocytes by treatment with insulin instead of progesterone. Insulin-induced meiosis of *Xenopus* oocytes apparently requires the activity of farnesyl transferase, the enzyme which transfers the farnesol isoprenyl group to particular proteins, since microinjection of specific peptides, which act as inhibitors of this activity, block GVBD (Chung et al., 1992). The protein(s) that become farnesylated in response to insulin, which act to trigger GVBD, have not been identified, but are suspected to be ras-like proteins. The activity of endogenous ras is thought to be required for insulin-induced GVBD, but not progesterone-induced GVBD, since microinjection of monoclonal antibodies to ras can inhibit GVBD after insulin treatment but not after progesterone treatment (Deshpande and Kung, 1987; Korn et al., 1987). In addition, microinjection of oncogenic ras protein into oocytes induces GVBD and meiosis (Birchmeier et al., 1985; Allende et al., 1988). The isoprenoid modification of ras is, apparently, functionally important in this activity since coinjection of compactin blocks ras-induced, but not progesterone-induced, GVBD (Schafer et al., 1989). Thus, the requirement of farnesylation activity for insulin-induced GVBD may be a requirement for an active ras-like protein, which is farnesylated after insulin treatment, and for which this modification is necessary for function.

There may be a similar type of requirement for the activation of clam oocytes. The release of clam oocytes from cell cycle arrest by fertilization or KCl treatment apparently requires 3–6 min of HMG-R activity. The requirement for HMG-R for this process perhaps is due to a requirement for synthesis of the isoprenoid that is used for modification of a protein(s), which may be ras-like, at the time of activation. In such a scenario, since new protein synthesis is not required for activation in clams (Hunt et al., 1992), this protein would have to be premade and stored in a non-isoprenylated form in the oocyte. Fertilization or KCl treatment would act to trigger the synthesis and attachment of the isoprenoid modification of this protein, which would then be functionally able to initiate the process of activation. While this hypothesis is useful in that it provides a model that can be tested experimentally, it should be noted that, in those cases where it has been studied, protein prenylation appears to occur on targeted proteins directly after translation (see Clarke, 1992) and has not been seen to serve in a regulatory role for protein function.

A required, early event for clam oocyte activation is the influx of calcium from the external environment (Allen, 1953). The influx of calcium after fertilization or KCl treatment is thought to be mediated through the opening of voltage-gated channels by a transient depolarization (Finkel and Wolf, 1980). However, these channels have not been identified in the clam, nor have the mechanisms by which they are regulated been elucidated. The influx of Ca^{++} in mast cells after stimulation, which is required for the activation of downstream signaling responses involved in inflammatory mediator secretion, is inhibited by incubation with HMG-R inhibitors (Deanin et al., 1991). This inhibition is overcome by mevalonate but not by sterols, suggesting that a non-sterol mevalonate-derived compound is important, although this compound has not been identified, nor has the manner in which it influences the influx of Ca^{++}. It may be that similar compounds act to mediate the influx of Ca^{++} in clam oocytes after KCl treatment or fertilization, a model that is experimentally testable.

Requirements of HMG-R Activity for Meiotic Cell Cycle Progression

In activated clam oocytes, treatment with inhibitors of HMG-R induces a block to those processes that result in cyclin proteolysis at metaphase I. Using cell-free systems in which programmed events of the mitotic cell cycle are faithfully reproduced, it has been shown that the cell cycle–regulated degradation of the cyclins occurs via ubiquitin-mediated proteolysis (Glotzer et al., 1991; Hershko et al., 1991). Ubiquitin-mediated proteolysis of non-cyclin proteins appears to occur as well at the metaphase/anaphase transition in mitosis, and is required for the separation of sister chromatids during anaphase (Holloway et al., 1993). In clams, the enzymatic components of the ubiquitin system, which brings about cyclin destruction during mitosis, appear to be active during metaphase of meiosis I also (Hershko et al., 1994). The fact that inhibitors of HMG-R block the activation of cyclin proteolysis and anaphase in activated clam oocytes, suggests that metabolites from the mevalonate biosynthetic pathway may act upstream to influence either the activation of ubiquitin-mediated proteolysis during meiosis, or to render the cyclins, as well as other potential proteins, the destruction of which may be required for anaphase, available to the action of the enzymes involved in proteolysis. However, positive or negative influences of metabolites in the mevalonate biosynthetic pathway for the enzymatic steps of ubiquitin-mediated proteolysis have not been noted (for review see Hershko and Ciechanover, 1992).

HMG-R itself is an enzyme, the activity of which is tightly regulated at several levels including that of protein turnover (for review see Goldstein and Brown, 1990). The proteolytic mechanisms by which HMG-R are degraded have not been elucidated, however recent studies (Sherwood et al., 1993) indicate that there may be commonalities in the pathways of HMG-R proteolysis and those resulting in cyclin destruction. In these studies, it was shown that treatment of cells with the cysteine protease inhibitor, *N*-acetylleucylleucylnorleucinal, a known inhibitor of HMG-R proteolysis (Inoue et al., 1991) led to stabilization of cyclin B protein, with an accompanying stabilization of histone H1 kinase activity and metaphase arrest. It is of interest to note that proteolysis of HMG-R requires a non-sterol mevalonate derived compound and genetic studies in yeast indicate that this requirement may be for a farnesylated protein (Roitelman and Simoni, 1992; Hampton and Rine, 1994). There may be a requirement for a mevalonate-derived component necessary for cyclin destruction in activated clam oocytes during meiosis that may be similar to that needed for the pathway of HMG-R proteolysis.

Requirements of HMG-R Activity for Cell Cycle Progression in Mitotically Dividing Cells

Inhibition of mevalonate synthesis in a variety of mitotically dividing postembryonic cell types induces an arrest in the G1 phase of the cell cycle (Cornell et al., 1977; Kaneko et al., 1978; Quesney-Huneeus et al., 1979; Habenicht et al., 1980; Perkins et al., 1982; Fairbanks et al., 1984; Sinensky and Logel, 1985; Doyle and Kandutsch, 1988; Chakrabarti and Engleman, 1991; Keyomarsi et al., 1991). In contrast to the effects seen in postembryonic cells, the addition of HMG-R inhibitors to early clam embryos does not affect their ability to divide mitotically. This finding is similar to what is seen for sea urchin embryos where progress through the early mitotic divisions is apparently unaffected by inhibition of HMG-R (Carson and Lennarz, 1979). In these cells, no affects of inhibiting HMG-R are noted until the time of gastrulation, which occurs abnormally or, for some embryos, is blocked.

Why would inhibition of HMG-R affect the passage through the two meiotic divisions, but not for the mitotic divisions? It may be that the activity of HMG-R increases in clam embryos during mitosis, and so would still be active in the presence of inhibitor concentrations that would block meiosis. Such changes of HMG-R activity occurs in sea urchin embryos where there is a dramatic increase during early developmental stages (Woodward, 1988 #327). Alternatively, there may be differential requirements of HMG-R activity for meiosis and mitosis. One major difference between the two meiotic divisions and the mitotic divisions is the origin of the microtubule organizing centers (MTOCs), which are involved in producing the spindle poles. The MTOCs which form the poles of the meiotic spindle are maternally derived, whereas those involved in the formation of the mitotic spindle are brought in with the sperm at the time of fer-

tilization (Longo and Anderson, 1970). In addition, the mechanism of spindle formation during meiosis may be different than during mitosis. This is suggested by studies in which differential effects on meiotic and mitotic spindles are seen following treatments with taxol, a microtubule-stabilizing agent (Kuriyama, 1986). It may be that metabolites from the mevalonate biosynthetic pathway influence the formation of the spindle during meiosis, but not mitosis.

Microtubule Polymerization, Spindle Formation, and Feedback Controls

Postembryonic cells have in place a checkpoint mechanism that prevents the exit from M phase until the chromosomes are aligned properly on the metaphase plate of the spindle (for review see Murray, 1992). This checkpoint is enacted in cells that are treated with agents, which disrupt the spindle, thereby preventing proper chromosome alignment at metaphase. Such treatments bring about a cell cycle arrest at metaphase accompanied by the stabilization of cyclin B and $p34^{cdc2}$ kinase activity. In clam embryos, this checkpoint appears to operate, since treatment with microtubule depolymerizing agents to disrupt the spindle results in a metaphase arrest of either meiosis or mitosis (Hunt et al., 1992 and K. Swenson, unpublished).

Inhibition of HMG-R in activated clam oocytes to induce a metaphase arrest at meiosis I does not appear to affect microtubule polymerization, spindle formation, or chromosome alignment. This indicates that the metaphase arrest in these cells is not being triggered by alterations of these processes, at least at a gross level. In other cell types as well, microtubule polymerization does not appear to be affected by HMG-R inhibitors (Fenton et al., 1992).

When activated clam oocytes arrested at metaphase I by HMG-R inhibition are fixed at 50 min postactivation, a time when control cultures are undergoing anaphase of meiosis II, the spindles are quadripolar instead of bipolar. If spindle pole splitting occurred normally in the absence of anaphase or cytokinesis (Kuriyama et al., 1986), the formation of a quadripolar spindle at 50 min postactivation would be expected. Our results suggest that HMG-R inhibition of activated clam oocytes, while blocking anaphase and cytokinesis, does not affect the normal splitting of spindle poles. Treatments which delay progress through mitosis in other cell types have been seen to induce multipolar spindles as well (Keryer et al., 1984; Sluder and Begg, 1985; Gallant and Nigg, 1992).

In yeasts, mutants of genes that function in mediating the checkpoint that operates as a feedback control during mitosis have been isolated (Hoyt et al., 1991; Li and Murray, 1991). These mutants were obtained in screens as ones which fail to delay the onset of anaphase in the presence of spindle damage caused by the microtubule depolymerizing drug, benomyl. To date, none of the products encoded by the genes which are affected by these mutations appear to be regulated by metabolites of the mevalonate biosynthetic pathway. Our results suggest that, during clam meiosis, either the build-up of metabolites from this pathway upstream of mevalonate, or the absence of a mevalonate-derived metabolite, is negatively influencing passage through the metaphase/anaphase transition. It may be that the metaphase arrest that occurs in these cells is due to the triggering of checkpoint control processes.

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