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Role of Maturation-Promoting Factor (p34^{cdc2}-Cyclin B) in Differential Expression of the *Xenopus* Oocyte and Somatic-Type 5S RNA Genes

VERONICA J. WOLF,[1] TAM DANG,[2] PHILIPPE HARTL,[2]† AND JOEL M. GOTTESFELD[1]*

Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037,[1] and Department of Biology, University of California, San Diego, La Jolla, California 92093-0322[2]

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Transcription of 5S rRNA and tRNA genes by RNA polymerase III (pol III) in cytosolic extracts of unfertilized *Xenopus* eggs and in a reconstituted system derived from *Xenopus* oocytes is repressed by the action of one or more mitotic protein kinases. Repression is due to the phosphorylation of a component of the pol III transcription apparatus. We find that the maturation/mitosis-promoting factor kinase (MPF, p34^{cdc2}-cyclin B) can directly mediate this repression in vitro. Affinity-purified MPF and immune complexes formed with antibodies to the protein subunits of MPF (p34^{cdc2} and cyclin B) retain both histone H1 kinase activity and the capacity to repress transcription in the reconstituted transcription system. Transcription complexes of oocyte-type 5S RNA genes and tRNA genes are quantitatively more sensitive to MPF repression than the corresponding transcription complexes of the somatic-type 5S RNA gene. The differential transcription of oocyte- and somatic-type genes observed during early *Xenopus* embryogenesis has been reproduced with the reconstituted transcription system and affinity-purified MPF. This differential transcription may be due to the instability of transcription complexes on the oocyte-type genes and the heightened sensitivity of soluble transcription factors to inactivation by mitotic phosphorylation. Our results suggest that MPF may play a role in vivo in the establishment of the embryonic pattern of pol III gene expression.

The developmental regulation of transcription of the 5S RNA genes of the amphibian *Xenopus laevis* has been a subject of intensive investigation for almost 20 years (reviewed in reference 53). The *Xenopus* genome contains several multigene families that encode the 120-base-long 5S rRNA; the major oocyte-type gene family contains 20,000 copies per haploid genome, whereas the somatic-type gene family contains only 400 copies per genome equivalent (34). 5S genes of both families require similar sets of transcription factors for synthesis of 5S RNA by RNA polymerase III (pol III) in vitro (for reviews, see references 14, 32, and 50). These factors include the 5S gene-specific zinc finger protein TFIIIA and the general pol III factors TFIIIB and TFIIIC. TFIIIA binds to the internal control region of the 5S genes and directs the assembly of the other components of the transcription complex. TFIIIC is a large multisubunit protein which binds directly to the B-block promoter element of tRNA-like pol III genes. For the 5S genes (which lack a B-block sequence), TFIIIC binds to the TFIIIA-5S gene binary complex primarily through protein-protein interactions (18). TFIIIB has recently been shown to consist of the TATA-box binding protein and pol III-specific TATA-binding protein-associated factors (21, 26, 45, 47). TFIIIB does not productively bind pol III templates in the absence of the other transcription factors. In the case of yeast 5S genes, TFIIIA and TFIIIC are assembly factors for the recruitment of TFIIIB, and TFIIIB is the true initiation factor for directing RNA pol III to the committed template (20).

During oogenesis, both the oocyte- and somatic-type gene families are actively transcribed. This is true both for the endogenous chromosomal 5S genes and for cloned genes microinjected into oocyte nuclei. Extracts prepared from oocyte nuclei transcribe the somatic-type gene about fivefold more efficiently (on a per-gene basis) than the oocyte-type gene (52). We will refer to this somatic preference as an S:O preference of 5:1. This is also the S:O preference observed for microinjected genes (6, 8). Whole oocyte S-150 extracts exhibit a 50:1 to 100:1 S:O preference (28, 29, 33). This is due to the limitation of TFIIIC in these extracts (48) and to the lower affinity of oocyte-type genes than of somatic-type genes for this factor (22, 23). When oocytes mature in vivo or when cultured oocytes are induced to mature in vitro by incubation with progesterone, oocyte chromatin condenses and the oocyte nucleus (the germinal vesicle) breaks down. Concomitant with these dramatic changes in nuclear structure, transcription is repressed both on the endogenous chromosomal 5S genes and on microinjected genes (8, 54). Mature *Xenopus* eggs are transcriptionally quiescent, and transcription in vivo does not resume until well after fertilization, at the mid-blastula transition (MBT) stage of embryogenesis (31). At this stage, the endogenous genes transcribe equal amounts of oocyte- and somatic-type 5S RNA, giving rise to an S:O preference of 50:1 (46, 54). The oocyte-type genes are progressively inactivated with each cell division following the MBT such that by early neurula, the S:O preference reaches 1,000:1 (46, 54). This is also the S:O preference observed in somatic cells. Extracts prepared from unfertilized eggs and early-stage embryos also show preferential transcription of the somatic-type genes, and under certain experimental conditions, the S:O preference can reach 400:1 (52). The molecular mechanisms responsible for these changes in 5S gene expression have not been fully elucidated.

We recently reported that mitotic repression of transcription can be reproduced in vitro for genes transcribed by RNA pol III (16, 17). Transcriptionally active interphase cytosol extracts

* Corresponding author. Mailing address: Department of Molecular Biology, The Scripps Research Institute, 10666 N. Torrey Pines Rd., La Jolla, CA 92037. Phone: (619) 554-8461. Fax: (619) 554-6665.

† Present address: Hormone Research Institute, University of California, San Francisco, San Francisco, CA 94143-0534.

of *Xenopus* eggs can be converted to the mitotic state by the addition of bacterially expressed B1 cyclin protein (30, 42). Cyclin complexes with the $p34^{cdc2}$ kinase present in the extract, and after specific phosphorylation and dephosphorylation events, the active form of maturation/mitosis-promoting factor (MPF) is generated (11, 19, 42, 43). Mitotic conversion of the extract represses pol III transcription from preformed transcription complexes (17). Our results further showed that transcriptional repression in vitro did not require chromosome condensation or nucleosome assembly of the template but rather could be effected by the direct phosphorylation of a component of the general pol III initiation factor TFIIIB (16, 17). We also showed that a secondary kinase, which was activated by mitotic conversion of the extract, could also mediate repression in vitro.

As oocyte maturation requires the action of the MPF protein kinase (10, 13, 27), we have now examined the possible direct role of this kinase in repression and in establishment of the oocyte-somatic switch in 5S gene expression. We find that highly purified MPF represses transcription in a reconstituted pol III transcription system and that this kinase can indeed reproduce differential 5S gene expression with cloned 5S genes and the reconstituted transcription system. Our results suggest that MPF may be involved in vivo in the establishment of differential expression of the two 5S gene families.

MATERIALS AND METHODS

DNA templates. The following plasmids containing RNA pol III-transcribed sequences from *X. laevis* have been described in detail elsewhere: the somatic-type 5S RNA gene (pXls 11 [34]), the oocyte-type 5S RNA gene (pXloΔ3′+176 [4]), synthetic oocyte- and somatic-type 5S genes lacking natural flanking sequences (36), the $tRNA^{Met1}$ gene (7), *tyrD* and *tyrC* tRNA genes (44), and pE190 containing one repeat unit of satellite I DNA (25). The predominant transcript of the synthetic 5S gene is 194 nucleotides in length as a result of a mutation at the natural terminator sequence (36). This allows discrimination between transcripts of the wild-type genes (121 nucleotides) and synthetic genes in the same reactions on denaturing polyacrylamide gels. Ratios of transcripts per gene in these reactions are corrected for the larger number of G residues in the longer transcripts (36 in the 121-nucleotide-long somatic-type 5S RNA and 51 in the 194-nucleotide-long transcript of the synthetic genes).

Transcription factors and reaction conditions. Partial purification of transcription factors TFIIIB and TFIIIC and RNA pol III from oocyte S-150 extracts (29) was carried out by using phosphocellulose chromatography as previously described (23, 38). TFIIIC was further purified by B-block DNA-Sepharose affinity chromatography (23) and contained 50 fmol of B-block DNA oligonucleotide binding activity per μl. TFIIIA was purified from immature oocytes as described previously (29). TFIIIB was further fractionated by sequential chromatography on DEAE-Sephadex and Mono Q fast protein liquid chromatography as described previously (16). Transcription reaction conditions were as described previously (23). Briefly, each reaction mixture contained, in a final volume of 20 μl, 12 μl of the 0.35 M KCl phosphocellulose fraction containing TFIIIB and RNA pol III, 2 μl of TFIIIC, and for the 5S RNA gene reactions, 9 to 18 ng of TFIIIA (generally yielding a 5- to 10-fold molar excess of TFIIIA over 5S genes). Nucleoside triphosphates were included at final concentrations of 0.6 mM for ATP, UTP, and CTP; GTP was included at a concentration of 0.02 mM along with 10 μCi of [α-^{32}P]GTP. Reactions were performed at final concentrations of 12 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-OH (pH 7.5), 60 mM KCl, 6 mM $MgCl_2$, 25 μM $ZnCl_2$, and 6 to 8% (vol/vol) glycerol (transcription buffer). Reactions were maintained for 2 h at ambient temperature, and RNA was purified and analyzed on denaturing polyacrylamide gels. In some experiments, RNAs were analyzed by electrophoresis on 0.35-mm-thick partially denaturing polyacrylamide gels (12.5% [wt/vol] at a 29:1 acrylamide-to-bisacrylamide ratio) containing 25% (wt/vol) urea (24, 33). These gels were prerun at 1,200 V for 2 to 4 h prior to electrophoresis for 15 h at 5 mA. This gel system separates oocyte- and somatic-type 5S RNAs of identical size (121 nucleotides) which differ in sequence at five positions. The difference in gel mobility of the two species of 5S RNAs is presumably due to differences in secondary structure under partially denaturing conditions (24). For quantitation of the relative transcription efficiencies of two or more templates in the same reactions, autoradiograms (taken within the linear range of the X-ray film) were scanned with an LKB laser densitometer.

Unfertilized egg extracts. Cytosol extracts from unfertilized *Xenopus* eggs were prepared as described previously (17, 41) and converted to the mitotic state by the addition of recombinant sea urchin Δ13 cyclin B1–glutathione *S*-transferase (GST) fusion protein (42, 43). Mitotic conversion was monitored by observing the breakdown of test nuclei added to the extract (42) and by induction of histone H1 kinase activity (see below).

Ammonium sulfate fractionation of interphase and mitotic extracts. Thirty percent ammonium sulfate precipitates from both the interphase and B1 cyclin-converted mitotic extracts were prepared as described previously (17, 41) except that precipitations were carried out with 200-μl aliquots of cytosol extracts, 200 μl of modified EB buffer (100 mM β-glycerolphosphate, 7.5 mM $MgCl_2$, 2 mM EGTA, 1 mM dithiothreitol, 10% [vol/vol] glycerol, 10 mM Tris-Cl [pH 7.5]) and 0.43 volume of 3.6 M ammonium sulfate (in EB buffer). Precipitates were allowed to form on ice for 30 min and were collected by centrifugation in a microcentrifuge at 4°C for 30 min. Pellets were redissolved in 200 μl of EB buffer and were dialyzed against EB buffer with a Pierce microdialyzer at 4°C. The protein concentrations of these fractions ranged from 4 to 7 mg/ml. The histone H1 kinase activities of the resulting ammonium sulfate fractions were monitored as described below.

Immunoprecipitations and binding of kinases to p13-agarose and glutathione-Sepharose. The following rabbit polyclonal antisera and preimmune sera were coupled to protein A-Sepharose 6MB (Pharmacia): antipeptide antibodies to $p34^{cdc2}$ (PSTAIRE; Santa Cruz Biotechnology), anti-C-terminal peptide antibodies to *Xenopus* B1 and B2 cyclins (a gift of D. Donoghue, University of California, San Diego), antibody to B1 cyclin (47a), and antibody to p42 mitogen-activated protein (MAP) kinase (a gift of M. Cobb, University of Texas Southwestern Medical School, Dallas). Protein A-Sepharose was washed several times with transcription buffer (see above), and equal volumes of packed Sepharose, antiserum, and buffer were incubated overnight at 4°C on a rotator. Unbound serum proteins were removed by repeated washings with transcription buffer. These protein A-Sepharose beads were then used to bind their target antigens present in the mitotic extract. Equal volumes of mitotic extract and packed beads (generally 20 μl) were incubated on the rotator for 1 h, and unbound proteins were again removed by repeated washings with transcription buffer. p13-agarose beads were purchased from Oncogene Science and washed three times with transcription buffer prior to incubation with an equal volume of either the mitotic or interphase egg cytosol extracts as described above. A similar

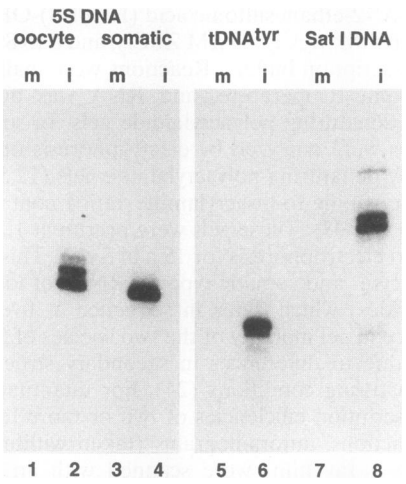

FIG. 1. Transcription of pol III genes in mitotic and interphase unfertilized egg cytosol extracts. Extracts from unfertilized *Xenopus* eggs were converted to the mitotic state by the addition of 1/10 volume of Δ13 cyclin B1-GST fusion protein (at 400 ng/μl). Lanes m, mitotic extracts; lanes i, interphase extracts. Reactions of lanes 1 and 2 contained 500 ng of plasmid pXloΔ3′+176 DNA containing the *X. laevis* oocyte-type 5S RNA gene; reactions of lanes 3 and 4 contained 200 ng of plasmid pXls11 containing the somatic-type 5S RNA gene; reactions of lanes 5 and 6 contained 200 ng of a plasmid containing the *X. laevis* $tRNA^{TyrD}$ gene; and reactions of lanes 7 and 8 contained 200 ng of plasmid pE190 containing satellite I of *X. laevis*. Products of transcription were analyzed on a denaturing polyacrylamide gel, and the autoradiogram is shown.

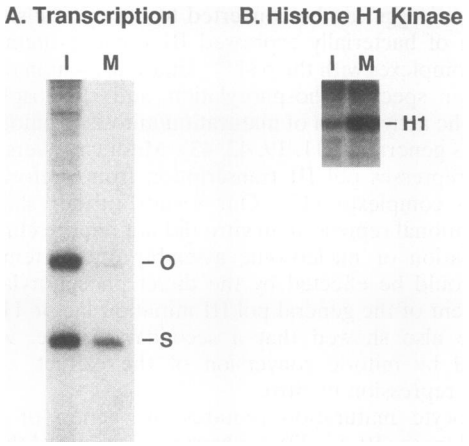

FIG. 2. Differential oocyte- and somatic-type 5S gene transcription after conversion of the interphase extract to the mitotic state. (A) A 5:1 mixture of oocyte-type (300 ng) and somatic-type (60 ng) 5S gene plasmid DNAs was preincubated with a 10-fold molar excess of TFIIIA prior to the addition of 25 μl of interphase cytosol to each reaction. After a subsequent 30-min incubation, 3 μl of recombinant B1 cyclin (1.2 μg) was added for the reaction of lane M. An equivalent volume of buffer was added for the reaction of lane I. After a subsequent 30-min incubation, [α-^{32}P]GTP was added, and reactions were terminated after a subsequent 2-h incubation. Transcripts from the synthetic oocyte-type gene (o; 194 nucleotides) and the natural somatic-type gene (s; 121 nucleotides) were resolved on a denaturing gel. (B) Aliquots of the interphase and mitotic extracts from the transcription assays shown in panel A were tested for histone H1 kinase activity. The autoradiogram of an SDS-PAGE analysis is shown.

procedure was used to bind the activated p34^{cdc2}-cyclin B1-GST fusion protein to glutathione-Sepharose (Pharmacia) except that XB buffer (100 mM KCl, 50 mM sucrose, 10 mM HEPES-OH [pH 7.7], 1 mM $MgCl_2$, 0.1 mM $CaCl_2$) was used. In some experiments, the bound kinase was eluted from glutathione-Sepharose with a buffer containing 10 mM HEPES-OH (pH 8.0), 1 mM dithiothreitol, and 5 mM glutathione (freshly prepared and adjusted to pH 8.0). Binding of protein kinases to p13-agarose, glutathione-Sepharose, or protein A-Sepharose-antibody conjugates was confirmed by phosphorylation of histone H1 with [γ-^{32}P]ATP (see below).

Protein phosphorylation. Histone H1 (Boehringer Mannheim) phosphorylation assays were performed with p13-agarose- or glutathione-Sepharose-bound proteins, with immunoprecipitates from the mitotic egg extract, with aliquots taken from transcription reactions, and with proteins eluted from glutathione-Sepharose. Immunoprecipitates, p13-agarose-protein complexes, and glutathione-Sepharose-protein complexes were washed three times with 5 to 10 volumes of EB buffer prior to assaying for kinase activity. Histone phosphorylations were performed in 20-μl reactions with 1 μg of histone H1, 100 μM ATP, and 13 μCi of [γ-^{32}P]ATP in EB buffer for 1 h at room temperature. Samples were subjected to precipitation with 25% (vol/vol) trichloroacetic acid prior to analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For H1 kinase assays of affinity matrix-immobilized proteins, incubations were continued for 1 h at room temperature on a rotator. After this time, the beads were pelleted and washed once with EB buffer, and the combined supernatants were precipitated with trichloroacetic acid and analyzed by SDS-PAGE. Alternatively, histone H1 was bound to phosphocellulose paper (Whatman P81) prior to quantitation by scintillation counting (41).

RESULTS

Transcription of pol III genes in interphase and mitotic egg cytosol extracts. Interphase extracts prepared from unfertilized *Xenopus* eggs are highly active in transcription of various genes by RNA pol III (2, 17), including genes encoding *Xenopus* oocyte- and somatic-type 5S RNA, tRNAs, and the pol III transcription unit contained within *Xenopus* satellite I DNA (Fig. 1, lanes i). In contrast, mitotic extracts, generated by the addition of recombinant cyclin B1 protein to the interphase cytosol (17), are largely inactive in pol III transcription (Fig. 1, lanes m). In the experiment shown in Fig. 1, the interphase extract is converted to the mitotic state prior to the addition of the DNA template, and full repression of transcription is observed (>99%). We wished to know whether genes that are differentially expressed in vivo might show different qualitative or quantitative responses to mitotic protein kinases in vitro when these genes are preassembled into transcription complexes. When a 5:1 mixture of oocyte- and somatic-type 5S RNA genes is transcribed in the interphase cytosol extract, we observe a 5:1 to 7:1 S:O preference (Fig. 2A, lane I). In this experiment, we used a synthetic oocyte-type gene which lacks all natural flanking sequences (36). This oocyte-type gene fails to support proper termination of transcription, and the predominant in vitro transcript from this gene is 194 nucleotides in length (23). Thus, transcripts from this oocyte-type gene and the natural somatic-type gene can be distinguished on a standard denaturing polyacrylamide gel (Fig. 2A). When a reaction equivalent to that shown in lane I is converted to the mitotic state by the addition of recombinant cyclin (after an initial incubation period to allow transcription complexes to assemble), we then observe an ~50:1 S:O preference (lane M). This change in the S:O ratio is due to the nearly complete

repression of oocyte-type transcription (~95%) and only partial repression of somatic-type 5S transcription (~72 to 80%). These results suggest that the transcription complexes containing the somatic-type 5S genes are more resistant to the mitotic kinase activity than the corresponding transcription complexes of the oocyte-type genes. Figure 2B shows the results of a histone H1 phosphorylation experiment with aliquots taken from the reactions shown in Fig. 2A. Densitometry of the autoradiogram indicates that addition of recombinant cyclin to the interphase cytosol induces an ~10-fold-higher level of histone H1 kinase activity.

Repression of pol III transcription in vitro by a protein kinase fraction isolated from mitotic egg cytosol extracts. The data presented in Fig. 2 suggest that a quantitative difference exists between oocyte-type and somatic-type 5S gene transcription complexes in their responses to mitotic protein phosphorylation. To directly test whether genes that are differentially transcribed in vivo show differential sensitivity to mitotic kinases in vitro, we performed a titration experiment with increasing amounts of partially purified MPF added to preformed transcription complexes (Fig. 3). As a source of MPF, we used a 30% ammonium sulfate-insoluble protein fraction isolated from the mitotic extract; as a control, we used an equivalent protein fraction from the interphase extract. Transcription complexes were formed by incubating the DNA templates with a fractionated system consisting of partially purified transcription factors and RNA pol III for 30 min (see Materials and Methods). We then added the MPF fraction (or the control interphase fraction), and after incubation with ATP to allow protein phosphorylation, we assayed for transcriptional activity. Addition of the mitotic protein fraction (lanes 3 to 7) to transcription complexes assembled on a mixture of oocyte- and somatic-type 5S RNA genes and the $tRNA^{Met1}$ gene results in marked inhibition of transcription from each of these templates. (A partially denaturing polyacrylamide gel was used in this experiment to distinguish between the transcripts of the natural oocyte- and somatic-type 5S genes [24]). Additionally, we find that different pol III genes preassembled into active transcription complexes exhibit different quantitative responses to the mitotic kinase fraction. In agreement with our previous observations (17), the somatic-type 5S gene is repressed by about 80% by the mitotic fraction (relative to the interphase control), whereas both the oocyte-type 5S RNA gene and the oocyte-type $tRNA^{Met1}$ gene are repressed by greater than 95% in this assay. In the absence of the mitotic kinase fraction, the somatic-type 5S RNA gene has a sevenfold competitive advantage over the oocyte-type 5S gene (lane 2; S:O preference of 6.7 ± 0.8 [n = 7]). After incubation with the kinase fraction, this competitive advantage is increased to 50:1 (S:O preference of 49 ± 9 [n = 5]). This result quantitatively reproduces the differential transcription of the oocyte and somatic-type 5S genes observed during early embryogenesis.

Repression of transcription is due to protein phosphorylation. To demonstrate that the repression of transcription that we observe is due to the direct enzymatic action of a mitotic protein kinase, we examined the effect of the kinase inhibitor 6-dimethylaminopurine (DMAP) on repression. Figure 4 shows that the inhibitory effect of mitotic kinases on 5S RNA and $tRNA^{TyrD}$ transcription complexes is completely prevented by low concentrations of DMAP. Data are presented for the complete mitotic extract (the somatic-type 5S gene) and for the MPF fraction added to the reconstituted transcription system ($tRNA^{TyrD}$ gene). Figure 4 also shows that rescue of transcription with DMAP occurs at concentrations which also inhibit mitotic histone H1 kinase activity. These data strongly

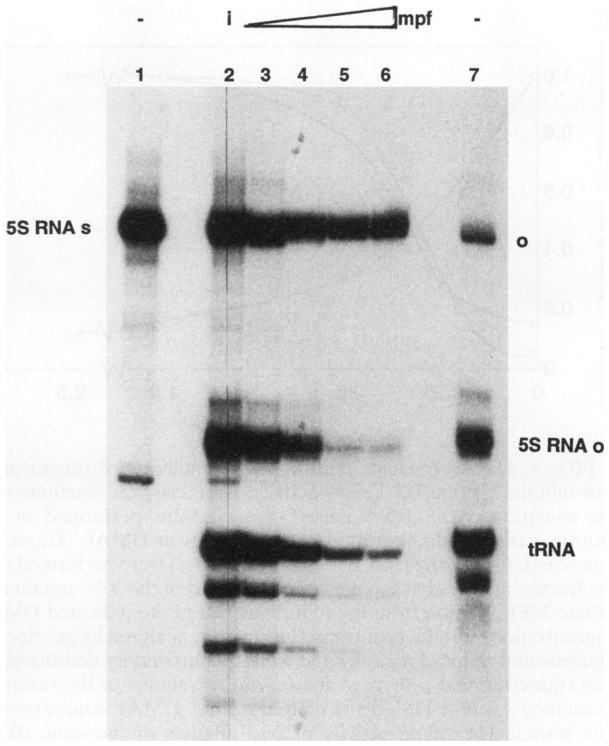

FIG. 3. Effect of a kinase-enriched protein fraction on RNA pol III transcription from preformed transcription complexes. Lane 1 shows the products of transcription of 20 ng of somatic-type 5S DNA and 100 ng of pUC19 carrier DNA; the reaction shown in lane 7 contained 100 ng of oocyte-type 5S DNA and 230 ng $tDNA^{Met1}$. All other reactions contained a mixture of these DNAs at the amounts listed above. Transcription complexes were formed by preincubation of the DNAs for 30 min with 18 ng of TFIIIA, TFIIIC, and the phosphocellulose TFIIIB-plus-RNA pol III fraction and unlabeled nucleoside triphosphates. For the reactions of lanes 1 and 7, 5 μl of EB buffer was added; for the reaction of lane 2, 5 μl of a 30% ammonium sulfate fraction from an interphase extract was added; for the reactions of lanes 3 to 6, 0.5-, 1-, 2-, and 5-μl aliquots of a 30% ammonium sulfate fraction from the mitotic extract (MPF) were added along with EB buffer to a final addition of 5 μl per reaction. After an additional incubation for 30 min, 10 μCi of [α-^{32}P]GTP was added to each reaction, and incubation was continued for an additional 2 h prior to isolation of the RNA transcripts and analysis on a partially denaturing gel. The upper band labeled o in lane 7 probably represents a readthrough transcript of the oocyte-type gene (4).

suggest that inhibition of transcription is due either directly or indirectly to protein phosphorylation by MPF.

We recently found that agarose beads coupled with the yeast $p13^{suc1}$ gene product bind the inhibitory activity from the mitotic extract (16) (Fig. 5A, lane 2). In this experiment, p13-agarose beads are first incubated with the mitotic extract and unbound proteins are removed by repeated washings. These beads are then incubated with a mixture of soluble transcription factors and pol III in the presence of ATP. The beads are removed by centrifugation, and the factors are then tested for the ability to support transcription. As p13 affinity resins have been shown to bind both the *cdc* and *cdk* kinases and, to a lesser extent, the MAP kinases (12, 40), we examined whether antibodies to components of MPF ($p34^{cdc2}$ and the B1 and B2 cyclins) could immunoprecipitate both histone H1 kinase activity and the inhibitor of transcription from the mitotic extract. Figure 5 shows the results of an experiment in

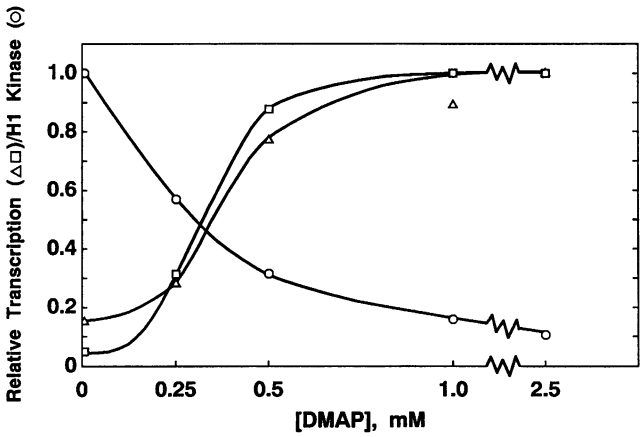

FIG. 4. DMAP prevents mitotic kinase inhibition of transcription and inhibits histone H1 kinase activity. Transcription reactions with the somatic-type 5S RNA gene (squares) were performed in the mitotic extract at the indicated concentrations of DMAP. Transcription reactions with the $tRNA^{TyrD}$ gene (triangles) were performed with the fractionated system supplemented with 2 μl of the 30% ammonium sulfate MPF fraction from the mitotic extract at the indicated DMAP concentrations. Products of transcription were analyzed by gel electrophoresis and autoradiography and were quantitated by densitometry. The transcriptional activity of the reactions (relative to the reactions containing 2.5 mM DMAP) is plotted against DMAP concentration. The histone H1 kinase activity of 2-μl aliquots of the same tRNA reactions relative to the 0 mM DMAP sample are also plotted against DMAP concentration (circles).

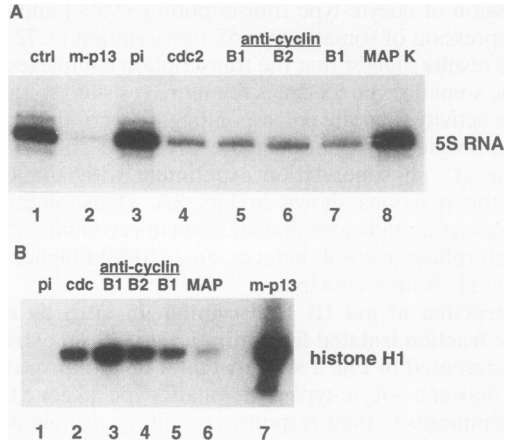

FIG. 5. Antibodies to MPF immunoprecipitate an inhibitor of transcription and histone H1 kinase activity. (A) p13-agarose (lane 2) and protein A-Sepharose-antibody complexes were used to absorb proteins from the mitotic extract. The following antibodies were bound to protein A-Sepharose: preimmune rabbit serum (lane 3), antibody to $p34^{cdc2}$ kinase (anti-PSTAIRE; lane 4), anti-B1 cyclin antipeptide antibody (lane 5), anti-B2 cyclin antipeptide antibody (lane 6), polyclonal antibody reactive with B1 cyclin (lane 7), and polyclonal anti-MAP kinase antibody (lane 8). After removing unbound proteins, the p13-agarose and protein A-Sepharose immune complexes were incubated with a mixture of TFIIIA, TFIIIB, TFIIIC, RNA pol III, and unlabeled nucleoside triphosphates. After 1 h on a rotator, the beads were pelleted and the supernatants were tested for transcription activity with the somatic-type 5S RNA gene (200 ng) and [α-^{32}P]GTP. A control reaction is shown in lane 1. (B) The pelleted beads were assayed for histone H1 kinase activity. The autoradiogram of an SDS-PAGE analysis is shown. The antibodies were as for panel A. Lane 3 is the anti-B1 cyclin antipeptide antibody, and lane 5 is the polyclonal serum reactive with B1 cyclin.

which protein A-Sepharose beads coupled with antibodies to $p34^{cdc2}$ (anti-PSTAIRE peptide antibodies), the B1 and B2 cyclins (antipeptide and polyclonal anti-B1 cyclin), and control antibodies (preimmune serum and anti-MAP kinase antibodies) were used to bind proteins from the mitotic extract. These immune complexes were then tested for histone H1 kinase activity (Fig. 5B) and for the capacity to inhibit the transcriptional activity of a mixture of factors and polymerase (Fig. 5A). Immune complexes formed with antibodies to the components of MPF retain both activities. In contrast, preimmune serum and anti-MAP kinase antibodies do not precipitate either H1 kinase or the inhibitory activity from the mitotic extract. (The anti-MAP kinase immunoprecipitates were active in phosphorylation of the MAP kinase substrate myelin basic protein [not shown].) p13-agarose beads retain far higher levels of H1 kinase activity (Fig. 5B, lane 7) and are more active in repressing transcription than immune complexes formed with any of the anti-MPF component antibodies (Fig. 5A; compare lane 2 with lanes 4 to 7). This could be due to a higher affinity of p13 for MPF or to the masking of the catalytic activity of MPF by the antibodies. Nonetheless, these results suggest that the $p34^{cdc2}$-cyclin B kinase is sufficient to initiate phosphorylation and thereby inactivate a soluble component(s) of the pol III transcription apparatus.

Affinity-purified $p34^{cdc2}$-cyclin B inhibits transcription and can establish differential oocyte-somatic 5S gene transcription. Since the recombinant cyclin B1 used to convert the interphase cytosol extract to the mitotic state is a GST fusion protein (43), we are able to isolate the activated $p34^{cdc2}$-cyclin B1 kinase from the extract via this GST tag. Histone H1 phosphorylation experiments confirm that the majority of the H1 kinase activity in the mitotic extract can be bound to glutathione-Sepharose and subsequently eluted from the resin with 5 mM glutathione (Fig. 6B). Both the glutathione-Sepharose-bound and unbound fractions repress 5S gene transcription when added to a mixture of transcription factors and polymerase simultaneously with the DNA template (Fig. 6A). The corresponding protein fractions from the interphase extract exhibit little H1 kinase activity and do not repress transcription. SDS-PAGE of the glutathione-Sepharose-bound proteins from the mitotic extract show only the GST-cyclin fusion protein, $p34^{cdc2}$, and bacterial glutathione-binding proteins as significant components of this fraction (not shown).

We next tested the effect of the glutathione-Sepharose-purified kinase on preformed transcription complexes. A 5:1 mixture of oocyte- and somatic-type 5S gene templates was incubated with the reconstituted transcription system for the times indicated in Fig. 7, and these reactions were then supplemented with the glutathione-Sepharose-purified proteins isolated from either the interphase or mitotic extracts. As before, both oocyte- and somatic-type 5S gene transcription is repressed by the mitotic kinase if the kinase is added at time zero (no preincubation of DNA and transcription factors; lane 4). If, however, the DNA templates are preincubated with factors for 30 or 60 min, a differential inhibitory effect is observed. In the absence of the mitotic kinase, the S:O preference is 6.7:1. In the presence of the affinity-purified MPF kinase, the S:O transcript ratio is 49:1 (average of five determinations at 30 to 60 min of preincubation). While the overall level of repression of both 5S genes appears to be reduced after prolonged preincubation times, the length of preincubation is without effect on the S:O transcript ratio. This suggests that differences in rates of transcription complex assembly (39)

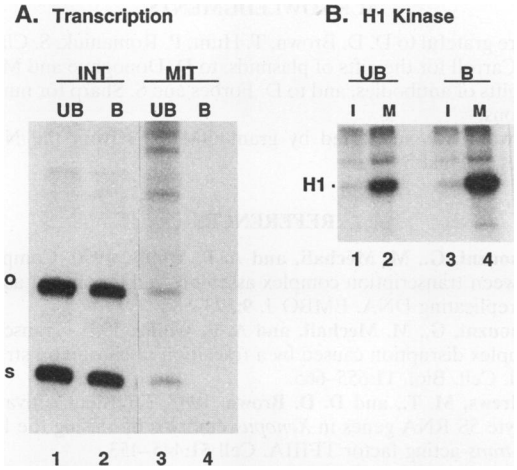

FIG. 6. p34^{cdc2}-cyclin B1 (GST fusion protein) kinase represses pol III transcription. Interphase (INT [A] or I [B]) and mitotic (MIT [A] or M [B]) cytosol extracts were subjected to chromatography on glutathione-Sepharose (as described in Materials and Methods), and 5-μl aliquots of both the unbound (UB) and bound (B) fractions were assayed for their effects on 5S gene transcription (A) and for histone H1 kinase activity (B). Transcription reactions contained 60 ng of pXls11 somatic-type 5S DNA and 300 ng of the synthetic oocyte-type gene plasmid DNA along with TFIIIA, TFIIIC, and the phosphocellulose TFIIIB-plus-RNA polymerase III fraction. Transcription components, unlabeled nucleoside triphosphates, DNA, and kinase fractions were incubated together for 1 h prior to the addition of [α-^{32}P]GTP. Produets of transcription were purified and analyzed after a subsequent 2-h incubation. The gel positions of the transcripts from the oocyte-type (o) and somatic-type (s) 5S gene templates are shown.

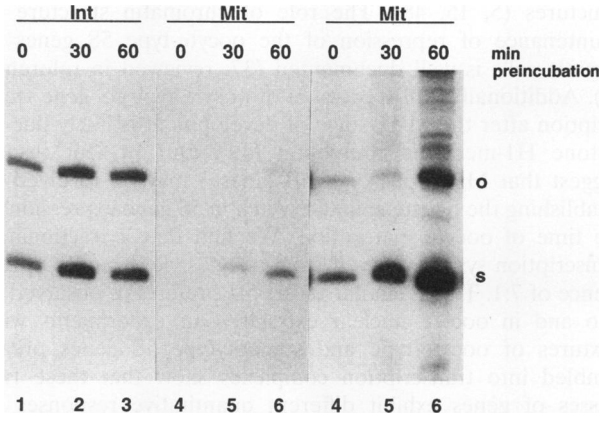

FIG. 7. p34-cyclin B1 kinase is sufficient to establish differential 5S gene transcription from preformed complexes. A 5:1 mixture of oocyte and somatic 5S gene plasmid DNAs (as in Fig. 6) was preincubated at ambient temperature with transcription fractions and unlabeled nucleoside triphosphates for the times indicated prior to the addition of 4 μl of glutathione-Sepharose-bound proteins isolated from either the interphase (Int; lanes 1 to 3) or mitotic (Mit; lanes 4 to 6) extract. After a subsequent 1-h incubation, [α-^{32}P]GTP was added, and transcripts were isolated after an additional 2-h incubation. Lanes 1 to 6 on the left show a 1-h autoradiographic exposure; lanes 4 to 6 on the right were from a 16-h exposure.

do not contribute to the differential responses of the oocyte- and somatic-type 5S genes to the *cdc2* kinase under our experimental conditions.

DISCUSSION

We have shown that activation of MPF by the addition of recombinant cyclin to a transcriptionally active interphase egg extract represses pol III transcription (17). This repression is due to the enzymatic action of a protein kinase, since inhibition can be blocked with the kinase inhibitor DMAP (Fig. 4) and inhibition requires a nucleoside triphosphate with a hydrolyzable β-γ phosphate bond (16). Depletion of MPF from a cyclin-converted mitotic egg extract by p13 affinity chromatography revealed the existence of a secondary kinase. This kinase was activated by MPF and, after activation, could repress transcription from preformed transcription complexes in the absence of MPF (17). Several lines of experimental evidence point to p34^{cdc2}-cyclin B as the kinase responsible for inhibition in our reconstituted transcription system: first, a 30% ammonium sulfate-insoluble fraction isolated from the mitotic extract contains H1 kinase activity (Fig. 4), the polypeptide constituents of MPF (data not shown), and the repressor of transcription (Fig. 3 and 4); second, we find that both H1 kinase activity and the repressor of transcription are retained on the p34^{cdc2} affinity resin p13-agarose (Fig. 5 and reference 16); third, immune complexes formed with protein A-Sepharose bound with antibodies to the components of MPF (p34 and cyclins B1 and B2) retain H1 kinase activity and the transcriptional repressor (Fig. 5); and fourth, highly purified p34-cyclin B-GST fusion protein (isolated by glutathione affinity chromatography) also retains these activities (Fig. 6). At present, we cannot exclude the possible existence of a secondary kinase activity in one of our transcription factor fractions.

We find that soluble pol III transcription factors are fully inactivated by mitotic protein phosphorylation (Fig. 1 and 6), whereas preformed transcription complexes on oocyte- and somatic-type pol III genes show differential sensitivities to MPF (Fig. 2, 3, and 7). Previous studies have shown that sequence differences within the internal control region of the oocyte- and somatic-type 5S genes are responsible for differential expression of these genes in vitro (33, 35, 52) and in microinjection experiments (6, 8). We wished to know whether the difference in mitotic repression of oocyte- and somatic-type 5S gene transcription that we observe is due to sequence differences between the coding regions of these two genes or to differences in flanking sequences. The oocyte-type 5S gene has a very A+T-rich 5′ flanking sequence, while the somatic-type gene has a highly G+C rich 5′ flank (34). The data of Fig. 2 and 7 show that the oocyte-type gene lacking all natural flanking sequences is also more sensitive to MPF repression than the somatic-type 5S gene harbored within its natural flanking sequence. We repeated this experiment with a synthetic somatic-type gene (36) and the natural oocyte-type gene and again obtained the same relative effects of MPF on oocyte- and somatic-type transcription (data not shown). Thus, sequence differences within the coding sequences of the oocyte and somatic-type 5S genes account for the differential response to MPF.

Differential 5S gene transcription is likely due to a combination of factors: different affinities of the two gene types for common transcription factors (22, 23, 48), different rates of transcription complex assembly (39), differential stability of transcription complexes on oocyte- and somatic-type genes (52), and competition for limiting amounts of transcription factors (3). It has been proposed that genes that are programmed into active transcription complexes escape repression by nucleosome assembly but those genes lacking transcription complexes are assembled into stable inactive chromatin

structures (5, 15, 48). The role of chromatin structure in maintenance of repression of the oocyte-type 5S genes in somatic cells is well documented (37; reviewed in reference 53). Additionally, full repression of oocyte-type 5S gene transcription after the MBT stage of development is likely due to histone H1-mediated repression (49). Our present results suggest that MPF (p34-cyclin B kinase) may be involved in establishing the oocyte-somatic switch in 5S gene expression at the time of oocyte maturation. We find that a fractionated transcription system derived from oocytes yields an S:O preference of 7:1. This is similar to the 5:1 preference observed in vivo and in oocyte nuclear extracts. Our experiments with mixtures of oocyte-type and somatic-type 5S genes preassembled into transcription complexes show that these two classes of genes exhibit different quantitative responses to MPF. Transcription from the somatic-type gene is generally repressed by 80%, whereas transcription from the oocyte-type gene shows greater than 95% inhibition of transcription (Fig. 3 and 7). In the presence of MPF, we observe an S:O preference of 50:1 to 100:1. This is the S:O preference observed in vivo once transcription resumes at the MBT stage of embryogenesis (46, 54). An oocyte-expressed methionine tRNA gene (44) also shows the same sensitivity to MPF as the oocyte-type 5S RNA gene (Fig. 3).

We suggest the following model for the establishment of the oocyte-somatic switch: upon induction of MPF activity in oocytes by hormone stimulation, transcription is repressed by the direct phosphorylation of components of the transcription complex. During the early cleavage stage of embryogenesis, transcription remains repressed by the continued action of MPF. Once the cell cycle lengthens at MBT, and MPF is inactivated by cyclin degradation (9), transcription factors are dephosphorylated and transcription resumes for the somatic-type genes. During early embryogenesis, transcription complexes are lost from the oocyte-type genes. This could be due to unsuccessful competition for limiting amounts of transcription factors at successive rounds of DNA replication (15) and to the relative instability of the oocyte-type 5S gene transcription complexes compared with the somatic-type gene transcription complexes (52). This complex instability could be exacerbated during mitosis followed by transcription factor dilution with each cell division.

Transcription complexes on the somatic-type 5S genes are stable in unfertilized egg extracts, whereas oocyte-type genes do not form stable complexes (52). Similarly, with our fractionated system, we find that transcription complexes on the oocyte-type genes are not stable, whereas complexes on the somatic-type gene are stable (data not shown). Wolffe and Brown (52) presented evidence for an activity that destabilizes transcription complexes on the oocyte-type genes. The finding of unstable complexes with the fractionated system suggests that instability may be an intrinsic property of the oocyte-type genes. Additionally, Almouzni et al. (2) have demonstrated the displacement of TFIIIB from the somatic-type 5S gene in a chromatin template by the addition of Mg^{2+} and ATP to interphase extracts. This finding could represent activation of another kinase that could influence transcription complex stability. Perhaps significantly, the target of mitotic phosphorylation involved in repression of pol III transcription is a component of TFIIIB (16). Thus, if TFIIIB is not stably bound to the oocyte-type genes, these genes would then be more susceptible to MPF-mediated repression than the somatic-type genes.

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REFERENCES

1. **Almouzni, G., M. Méchali, and A. P. Wolffe.** 1990. Competition between transcription complex assembly and chromatin assembly on replicating DNA. EMBO J. **9**:573–582.
2. **Almouzni, G., M. Mechali, and A. P. Wolffe.** 1991. Transcription complex disruption caused by a transition in chromatin structure. Mol. Cell. Biol. **11**:655–665.
3. **Andrews, M. T., and D. D. Brown.** 1987. Transient activation of oocyte 5S RNA genes in *Xenopus* embryos by raising the level of the *trans*-acting factor TFIIIA. Cell **51**:445–453.
4. **Bogenhagen, D. F., and D. D. Brown.** 1981. Nucleotide sequences in *Xenopus* 5S DNA required for transcription termination. Cell **24**:261–270.
5. **Bogenhagen, D. F., M. Wormington, and D. D. Brown.** 1982. Stable transcription complexes of *Xenopus* 5S RNA genes: a means to maintain the differentiated state. Cell **28**:413–421.
6. **Brown, D. D., and M. S. Schlissel.** 1985. A positive transcription factor controls the differential expression of two 5S RNA genes. Cell **42**:759–767.
7. **Clarkson, S. G., M. L. Birnstiel, and V. Serra.** 1973. Reiterated transfer RNA genes of *Xenopus laevis*. J. Mol. Biol. **79**:391–410.
8. **Darby, M. K., M. T. Andrews, and D. D. Brown.** 1988. Transcription complexes that program *Xenopus* 5S RNA genes are stable *in vivo*. Proc. Natl. Acad. Sci. USA **85**:5516–5520.
9. **Draetta, G., F. Luca, J. Westendorf, L. Brizuela, J. Ruderman, and D. Beach.** 1989. cdc2 protein kinase is complexed with both cyclin A and B: evidence for proteolytic inactivation of MPF. Cell **56**:829–838.
10. **Dunphy, W. G., L. Brizuela, D. Beach, and J. Newport.** 1988. The *Xenopus* cdc2 protein is a component of MPF, a cytoplasmic regulator of mitosis. Cell **54**:423–431.
11. **Dunphy, W. G., and J. W. Newport.** 1988. Mitosis-inducing factors are present in a latent form during interphase in the *Xenopus* embryo. J. Cell Biol. **106**:2047–2056.
12. **Fang, F., and J. W. Newport.** 1991. Evidence that the G1-S and G2-M transitions are controlled by different cdc2 proteins in higher eukaryotes. Cell **66**:731–742.
13. **Gautier, J., C. Norbury, M. Lohka, P. Nurse, and J. Maller.** 1988. Purified maturation promoting factor contains the product of a *Xenopus* homolog of the fission yeast cell cycle control gene $cdc2^+$. Cell **54**:433–439.
14. **Geiduschek, E. P., and G. P. Tocchini-Valentini.** 1988. Transcription by RNA polymerase III. Annu. Rev. Biochem. **57**:873–914.
15. **Gottesfeld, J. M., and L. S. Blommer.** 1982. Assembly of transcriptionally active 5S RNA gene chromatin *in vitro*. Cell **28**:781–791.
16. **Gottesfeld, J. M., V. J. Wolf, D. J. Forbes, and P. Hartl.** 1994. Mitotic repression of RNA polymerase III transcription *in vitro* mediated by phosphorylation of a TFIIIB component. Science **263**:81–84.
17. **Hartl, P., J. Gottesfeld, and D. J. Forbes.** 1993. Mitotic repression of transcription *in vitro*. J. Cell Biol. **120**:613–624.
18. **Hayes, J., T. D. Tullius, and A. P. Wolffe.** 1989. A protein-protein interaction is essential for stable complex formation on a 5 S RNA gene. J. Biol. Chem. **264**:6009–6012.
19. **Jessus, C., and D. Beach.** 1992. Oscillation of MPF is accompanied by periodic association between cdc25 and cdc2-cyclin B. Cell **68**:323–332.
20. **Kassavetis, G. A., B. R. Braum, L. H. Nguyen, and P. G. Geiduschek.** 1990. *S. cerevisiae* TFIIIB is the transcription initiation factor proper of RNA polymerase III, while TFIIIA and TFIIIC are assembly factors. Cell **60**:235–245.
21. **Kassavetis, G. A., A. P. Joazeiro, M. Pisano, E. P. Geiduschek, T. Colbert, S. Hahn, and J. A. Blanco.** 1992. The role of the TATA-binding protein in the assembly and function of the multi-

subunit yeast RNA polymerase III transcription factor, TFIIIB. Cell **71**:1055–1064.
22. **Keller, H. J., P. J. Romaniuk, and J. M. Gottesfeld.** 1992. Interaction of *Xenopus* TFIIIC with the TFIIIA-5S RNA gene complex. J. Biol. Chem. **267**:18190–18198.
23. **Keller, H. J., Q. You, P. J. Romaniuk, and J. M. Gottesfeld.** 1990. Additional intragenic promoter elements of the *Xenopus* 5S RNA genes upstream from the TFIIIA-binding site. Mol. Cell. Biol. **10**:5166–5176.
24. **Korn, L. J., and J. B. Gurdon.** 1981. The reactivation of developmentally inert 5S genes in somatic nuclei injected into *Xenopus* oocytes. Nature (London) **289**:461–465.
25. **Lam, B. S., and D. Carroll.** 1983. Tandemly repeated sequences from *Xenopus laevis*. J. Mol. Biol. **165**:567–585.
26. **Lobo, S. M., M. Tanaka, M. L. Sullivan, and N. Hernandez.** 1992. A TBP complex essential for transcription from TATA-less but not TATA-containing RNA polymerase III promoters is part of the TFIIIB fraction. Cell **71**:1029–1040.
27. **Lohka, M. J., M. K. Hayes, and J. L. Maller.** 1988. Purification of maturation-promoting factor, an intracellular regulator of early mitotic events. Proc. Natl. Acad. Sci. USA **85**:3009–3013.
28. **McConkey, G. A., and D. F. Bogenhagen.** 1988. TFIIIA binds with equal affinity to somatic and major oocyte 5S RNA genes. Genes Dev. **2**:205–214.
29. **Millstein, L., P. Eversole-Cire, J. Blanco, and J. M. Gottesfeld.** 1987. Differential transcription of *Xenopus* oocyte and somatic-type 5 S genes in a *Xenopus* oocyte extract. J. Biol. Chem. **262**:17100–17110.
30. **Murray, A. W., M. J. Solomon, and M. W. Kirschner.** 1989. The role of cyclin synthesis in the control of maturation promoting factor activity. Nature (London) **339**:280–286.
31. **Newport, J. W., and M. W. Kirshner.** 1984. Regulation of the cell cycle during early *Xenopus* development. Cell **37**:731–742.
32. **Palmer, J. M., and W. R. Folk.** 1990. Unraveling the complexities of transcription by RNA polymerase III. Trends Biochem. Sci. **15**:300–304.
33. **Peck, L. J., L. Millstein, P. Eversole-Cire, J. M. Gottesfeld, and A. Varshavsky.** 1987. Transcriptionally inactive oocyte-type 5S RNA genes of *Xenopus laevis* are complexed with TFIIIA in vitro. Mol. Cell. Biol. **7**:3503–3510.
34. **Peterson, R. C., J. L. Doering, and D. D. Brown.** 1980. Characterization of two *Xenopus* somatic 5S DNAs and one minor oocyte specific 5S DNA. Cell **20**:131–141.
35. **Reynolds, W. F.** 1988. Effect of sequence differences between somatic and oocyte 5S RNA genes on transcriptional efficiency in an oocyte S150 extract. Mol. Cell. Biol. **8**:5056–5058.
36. **Romaniuk, P. J.** 1988. The role of highly conserved single-stranded nucleotides of *Xenopus* 5S RNA in the binding of transcription factor IIIA. Biochemistry **28**:1388–1395.
37. **Schlissel, M. S., and D. D. Brown.** 1984. The transcriptional regulation of *Xenopus* 5S RNA genes in chromatin: the role of active stable transcription complexes and histone H1. Cell **37**:903–913.
38. **Segall, J., T. Matsui, and R. G. Roeder.** 1980. Multiple factors are required for the accurate transcription of purified genes by RNA polymerase III. J. Biol. Chem. **255**:11986–11991.
39. **Seidel, C. W., and L. J. Peck.** 1992. Kinetic control of 5 S RNA gene transcription. J. Mol. Biol. **227**:1009–1018.
40. **Shibuya, E. K., T. G. Boulton, M. H. Cobb, and J. V. Ruderman.** 1992. Activation of p42 MAP kinase and the release of oocytes from cell cycle arrest. EMBO J. **11**:3963–3975.
41. **Smythe, C., and J. W. Newport.** 1991. Systems for the study of assembly, DNA replication, and nuclear breakdown in *Xenopus laevis* egg extracts. Methods Cell Biol. **35**:449–468.
42. **Smythe, C., and J. W. Newport.** 1992. Coupling of mitosis to the completion of S phase in *Xenopus* occurs via modulation of the tyrosine kinase that phosphorylates $p34^{cdc2}$. Cell **68**:787–797.
43. **Solomon, M. J., M. Glotzer, T. H. Lee, M. Philippe, and M. W. Kirschner.** 1990. Cyclin activation of $p34^{cdc2}$. Cell **63**:1013–1024.
44. **Stutz, F., E. Gouilloud, and S. G. Clarkson.** 1989. Oocyte and somatic tyrosine tRNA genes in *Xenopus laevis*. Genes Dev. **3**:1190–1198.
45. **Taggart, A. K. P., T. S. Fisher, and B. F. Pugh.** 1992. The TATA-binding protein and associated factors are components of pol III transcription factor TFIIIB. Cell **71**:1015–1028.
46. **Wakefield, L., and J. B. Gurdon.** 1983. Cytoplasmic regulation of 5S genes in nuclear-transplant embryos. EMBO J. **2**:1613–1619.
47. **White, R. J., and S. P. Jackson.** 1992. Mechanism of TATA-binding protein recruitment to a TATA-less class III promoter. Cell **71**:1041–1053.
47a. **Wolf, V. J., and J. M. Gottesfeld.** Unpublished data.
48. **Wolffe, A. P.** 1988. Transcription fraction TFIIIC can regulate differential *Xenopus* 5S RNA gene transcription *in vitro*. EMBO J. **7**:1071–1079.
49. **Wolffe, A. P.** 1989. Dominant and specific repression of *Xenopus* oocyte 5S RNA genes and satellite I DNA by histone H1. EMBO J. **8**:527–537.
50. **Wolffe, A. P.** 1991. RNA polymerase III transcription. Curr. Biol. **3**:461–466.
51. **Wolffe, A. P.** 1993. Replication timing and *Xenopus* 5S RNA gene transcription *in vitro*. Dev. Biol. **157**:224–231.
52. **Wolffe, A. P., and D. D. Brown.** 1987. Differential 5S RNA gene expression *in vitro*. Cell **51**:733–740.
53. **Wolffe, A. P., and D. D. Brown.** 1988. Developmental regulation of two 5S ribosomal RNA genes. Science **241**:1626–1632.
54. **Wormington, W. M., and D. D. Brown.** 1983. Onset of 5 S RNA gene regulation during *Xenopus* embryogenesis. Dev. Biol. **99**:249–257.