# Mitotic Repression of Transcription In Vitro

Philippe Hartl,* Joel Gottesfeld,‡ and Douglass J. Forbes*

*Department of Biology, 0322, University of California at San Diego, La Jolla, California 92093-0322; ‡Department of Molecular Biology, The Scripps Research Institute, 10666 N. Torrey Pines Road, La Jolla, California 92037

*Abstract.* A normal consequence of mitosis in eukaryotes is the repression of transcription. Using *Xenopus* egg extracts shifted to a mitotic state by the addition of purified cyclin, we have for the first time been able to reproduce a mitotic repression of transcription in vitro. Active RNA polymerase III transcription is observed in interphase extracts, but strongly repressed in extracts converted to mitosis. With the topoisomerase II inhibitor VM-26, we demonstrate that this mitotic repression of RNA polymerase III transcription does not require normal chromatin condensation. Similarly, in vitro mitotic repression of transcription does not require the presence of nucleosome structure or involve a general repressive chromatin-binding protein, as inhibition of chromatin formation with saturating amounts of non-specific DNA has no effect on repression. Instead, the mitotic repression of transcription appears to be due to phosphorylation of a component of the transcription machinery by a mitotic protein kinase, either cdc2 kinase and/or a kinase activated by it. Mitotic repression of RNA polymerase III transcription is observed both in complete mitotic cytosol and when a kinase-enriched mitotic fraction is added to a highly simplified 5S RNA transcription reaction. We present evidence that, upon depletion of cdc2 kinase, a secondary protein kinase activity remains and can mediate this in vitro mitotic repression of transcription.

IN the early 1960s, it was observed that nuclear transcription in cultured cells becomes inhibited during mitosis. Specifically, incorporation of RNA precursors was found to cease in late prophase and to resume in telophase (Prescott and Bender, 1962). Mitotic repression of transcription was subsequently observed in many organisms, both for genes transcribed by RNA polymerase II and RNA polymerase III (Fink and Turnock, 1977; Johnston et al., 1987; White et al., 1987). Such mitotic repression of transcription would seem to benefit the cell in that untimely transcription, which might interfere with correct segregation of the replicated genome, is prevented. The mitotic repression of transcription has also been invoked as the explanation for the absence of transcription during early development of *Xenopus* and *Drosophila* embryos, where cells rapidly alternate between mitotic and S phase with no intervening G1 or G2 phase (Newport and Kirschner, 1984; edgar et al., 1986). Indeed, repression of transcription in early development can be alleviated simply by arresting the embryos in interphase with cycloheximide (Edgar and Schubiger, 1986; Kimelman et al., 1987). Although multiple hypotheses to account for the widely observed mitotic repression of transcription have been put forward, to date experiments designed to address different hypotheses have not been possible.

The potential mechanisms of mitotic repression include the following: First, it has been hypothesized that transcriptional silencing during mitosis is largely dependent on the condensation of the mitotic chromosomes (Johnson and Holland, 1965; Lewis et al., 1984). Condensation of chromosomes at mitosis is known to require topoisomerase II, which coincidentally becomes phosphorylated and increases in activity (Newport and Spann, 1987; Uemura et al., 1987; Adachi et al., 1991; Heck et al., 1989; Wood and Earnshaw, 1990; Hirano and Mitchison, 1991). A second hypothesis invokes a modification of nucleosome and/or chromatin structure at mitosis that would limit the accessibility of template DNA to transcription factors or RNA polymerase. This might be facilitated by the known mitotic hyperphosphorylation of the core histone H3 or the linker histone H1 (Bradbury et al., 1974; van Holde, 1988; Roberge et al., 1990; Bradbury, 1992). Numerous investigations have indicated that changes in chromatin structure during interphase can affect transcription (for reviews see van Holde et al., 1988; Felsenfeld and McGhee, 1986; Wolffe, 1991; Kornberg and Lorch, 1991; Felsenfeld, 1992). A third hypothesis involves the activation of a general repressive factor during mitosis. Such a factor would then bind to chromatin to prevent transcription. A mitotically phosphorylated histone H1 would also fit well into this model and, indeed, the presence of H1 can cause transcriptional repression in many interphase transcription extracts (Schlissel and Brown, 1984; Wolffe, 1989; Shimamura et al., 1989; Laybourn and Kadonaga, 1991). A fourth possibility is a direct and inhibitory phosphorylation of the transcriptional machinery itself at mitosis. Such phosphorylation could affect the DNA binding activity of specific

---

P. Hartl's present address is Hormone Research Institute, University of California, San Francisco, CA 94143-0534.

transcription factors or polymerase (Moreno and Nurse, 1990; Segil et al., 1991), or affect the initiation and elongation rates of transcription complexes. It has been observed in interphase cell extracts that the activity of transcription factors can be modulated by phosphorylation (Binetruy et al., 1991; Boyle et al., 1991; Roberts et al., 1991; Segil et al., 1991; for review see Corden, 1990), providing a precedent for such a mechanism during mitosis. RNA polymerase II has multiple potential phosphorylation sites in its COOH-terminus and can be phosphorylated in vitro by a *cdc2*-related kinase, although whether this is a mitotic or interphase kinase is as yet unknown (Cisek and Corden, 1989). Conceivably, in vivo more than one mechanism may operate to repress transcription. In any model, inhibition of transcription would be set in motion by entry into mitosis through the activation of the highly conserved mitotic kinase, mitosis promoting factor (MPF),[1] a complex of p34$^{cdc2}$ and cyclin B proteins (Dunphy et al., 1988; Gautier et al., 1988; Draetta et al., 1989; Solomon et al., 1990; for reviews see Lewin, 1990; Pines and Hunter, 1990).

Direct assessment of the mechanisms mediating transcriptional repression at mitosis requires an in vitro system that can accurately transcribe a template of interest. Ideally, this system should be able to be shifted between phases of the cell cycle. A system suited for these investigations is provided by extracts of *Xenopus* eggs, which when arrested in interphase are capable of accurate and efficient RNA polymerase III transcription and have been use to study the effect of interphase chromatin structure on transcription (Wolffe and Brown, 1987; Almouzni et al., 1990, 1991). In addition to interphase extracts, mitotic extracts can be made by the inclusion of EGTA during preparation and these are fully capable of nuclear disassembly and chromosome condensation (Lohka and Maller, 1985; Newport and Spann, 1987). Interphase extracts can be shifted directly to mitosis by the addition of either MPF or cyclin, the latter of which complexes with endogenous *cdc2* protein to produce MPF (Miake-Lye and Kirschner, 1985; Dunphy et al., 1988; Labbe et al., 1988; Minshull et al., 1989; Murray et al., 1989; Solomon et al., 1990). DNA, when injected into eggs or added to interphase extracts, initially becomes assembled into chromatin (Laskey et al., 1977; Dilworth et al., 1987); when the latter system is then converted to mitosis, the chromatin becomes condensed into a tightly packed structure reminiscent of mitotic chromosomes (Forbes et al., 1983; Hirano and Mitchison, 1991). Appropriate chromosomal proteins on the condensed chromatin also become phosphorylated in a mitotic manner in the converted extract (Hirano and Mitchison, 1991). Although to date no studies have addressed the mechanisms of mitotic repression of transcription, the *Xenopus* extracts appeared to us to have a strong advantage for approaching these questions.

Using *Xenopus* cell-free extracts we have been able to mimic for the first time mitotic repression of RNA polymerase III transcription in vitro. These studies have been carried out using two well-characterized genes transcribed by RNA polymerase III, those encoding the yeast tRNA$^{leu-3}$ and the *Xenopus* somatic 5S RNA (Geiduschek and Tocchini-

Valentini, 1988; Wolffe and Brown, 1988; Millstein and Gottesfeld, 1989). By manipulating the capacity of extracts to carry out full mitotic chromosome condensation, to form chromatin, or to potentially phosphorylate the transcriptional machinery, we have analyzed this mitotic repression. It appears likely that the latter mechanism is almost wholly sufficient to mediate transcriptional repression in vitro. Further investigations implicate an okadaic acid–sensitive protein phosphatase in antagonizing repression, and a secondary protein kinase, activated by but distinct from MPF, that can on its own mediate mitotic repression of transcription.

## Materials and Methods

### Template DNA

The tRNA$^{leu-3}$ gene, contained in plasmid pPC-1, was generously provided by E. Peter Geiduschek (University of California, San Diego, CA) and has been described by Kassavetis et al. (1989). The gene for *Xenopus* somatic 5S RNA, contained in plasmid pXlsl1, has also been described previously (Peterson et al., 1980). Plasmid DNA was transformed into *Escherichia coli* strain HB101.

### Reagents

Dimethylaminopurine (DMAP; Sigma Immunochemicals, St. Louis, MO) was dissolved in ultrapure DMSO (Pierce, Rockford, IL) at 300 mM immediately prior to use. VM-26 was generously provided as a 100 mM stock in DMSO by Allen Chen and Leroy Liu (Johns Hopkins University, Baltimore, MD) VM-26 stock solutions were stored at −70°C and diluted in DMSO immediately before use. Okadaic acid (Moana Bioproducts, Hawaii) was maintained as a 126-$\mu$m stock in water at −20°C until use (see Cohen, 1989, for a description of this inhibitor).

### Xenopus Egg Extract Preparation

*Xenopus* eggs were collected, dejellied, and lysed to prepare an interphase extract, as previously described (Smythe and Newport, 1991). Egg lysis buffer for the preparation of interphase extracts consisted of 250 mM sucrose, 50 mM KCl, 3 mM MgCl$_2$, 10 mM Hepes/NaOH, pH 7.5, supplemented with 1 mM DTT, 100 $\mu$g/ml cycloheximide, and 10 $\mu$g/ml aprotinin and leupeptin immediately before use. The added cycloheximide blocked protein synthesis, including endogenous cyclin synthesis, and thus maintained the extract in a stable interphase state. Crude extracts prepared by centrifugation at 10,000 rpm in a rotor (Sorvall HB-4; DuPont Co., Newton, CT) were supplemented with 5 $\mu$g/ml cytochalasin B. The crude extract was further separated into cytosol, membrane-rich, and gelatinous pellet fractions by ultracentrifugation at 200,000 g for 60 to 75 min in an ultracentrifuge (TL-100; Beckman Instruments, Inc., Fullerton, CA). The cytosol, used in the experiments reported here, was recentrifuged at 200,000 g for 30 minutes to remove residual membranes and glycogen (= clarified cytosol), divided into 25-$\mu$l aliquots, frozen in liquid nitrogen, and stored at −70°C until use. For a given experiment interphase aliquots were thawed and supplemented with an ATP-regenerating system (0.1 vol) at a final concentration of 2 mM ATP, 20 mM creatine phosphate, and 0.1 mg/ml creatine kinase.

Mitotic samples were generated by converting interphase cytosol (and ATP-regenerating system) to mitosis by the addition of one-tenth volume of recombinant Δ13 sea urchin cyclin B1 (1 to 2 mg/ml total protein). Δ13-cyclin was expressed in *E. coli* JM101 and affinity purified as described (Solomon et al., 1990; Smythe and Newport, 1992). Conversion was assayed by monitoring histone H1 kinase activity (MPF activity) and, in some cases, by the mitotic breakdown of test nuclei, as described in Smythe and Newport (1991).

### Transcription in Egg Extracts

Transcription reactions were carried out as shown in Fig. 1 A. For tRNA transcription reactions, template was added to clarified cytosol (supplemented with an ATP-regenerating system as described above) at a final concentration of 5 ng/$\mu$l. Carrier DNA (plasmid pBsIIKS+; Promega Biotec, Madison, WI) was simultaneously added to 15 ng/$\mu$l. 5S transcription

---

1. *Abbreviations used in this paper*: DMAP, dimethylamino-purine; MPF, mitosis promoting factor; PKA, protein kinase; TBP, TATA-binding protein; Topo II, topoisomerase II.

reactions contained 20 ng/μl template DNA which had been preincubated with 5 μl of TFIIIA at 18 ng/μl for 30 min. A typical transcription reaction contained 25 μl of cytosol and 1 μl of template DNA. When indicated in the individual figures, transcription reactions were mixed with the reagents shown and/or DNA, and then converted to mitosis with Δ13-cyclin. Interphase control transcription reactions were treated with cyclin buffer (XB; 50 mM sucrose, 100 mM KCl, 0.1 mM CaCl₂, 1 mM MgCl₂, 10 mM Hepes/KOH, pH 7.7) at a 1:10 dilution. Transcription was assayed starting 60 min after cyclin or buffer addition by adding α-$^{32}$P-GTP (5–10 μCi; Amersham Corp., Arlington Heights, IL). Unlabeled nucleotides were not added as they were already present in the extract at ~250 μM. Reactions were terminated 40 min later by addition of an equal volume of stop mix containing 1% SDS, 20 mM EDTA, 20% glycerol, and 50 mM Tris-Cl, pH 8.0. Proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN) was added to 1 mg/ml and the mixture incubated at 37°C for 1 h. Aliquots were then heated to 100°C and run on 4 M urea-0.1% SDS-10% polyacrylamide gels. After electrophoresis, gels were dried onto Whatman paper and exposed to Kodak ARO X-ray film (Eastman Kodak Co., Rochester, NY). Transcription was quantitated by densitometric scanning of autoradiographs or scintillation counting of bands excised from exposed gels.

In experiments where extracts were mixed with pl3-Sepharose, VM-26, or nonspecific DNA, interphase cytosol was first diluted by adding 0.4 vol of 100 mM β-glycerolphosphate, 20 mM Hepes/NaOH, pH 7.5, 10 mM MgCl₂, 100 mM KCl, and supplemented with the ATP-regenerating system described above. Dilution of the concentrated cytosol (20–30 mg/ml total protein) facilitated the handling of the extract for pl3-Sepharose treatment, reproducibility of chromatin formation experiments, and solubility of VM-26 without affecting the conversion to mitosis or repression of transcription. It should be noted that mitotic repression was not due to increased RNA degradation or a change in the GTP pool in the mitotic extract (data not shown).

## Ammonium Sulfate Fractionation of Interphase and Mitotic Extracts

30% ammonium sulfate precipitates of interphase and mitotic fractions were prepared essentially as previously described (Pfaller et al., 1991; Smythe and Newport, 1991). A single batch of interphase cytosol (3 ml) was divided into two aliquots. One batch was converted to mitosis with Δ13-cyclin; the other was treated with buffer XB. 60 min after cyclin or buffer addition, the extracts were diluted with an equal volume of standard EB buffer (80 mM β-glycerolphosphate, 15 mM EGTA, 15 mM MgCl₂, 1 mM DTT, pH 7.4) and mixed with 0.43 vol of 3.6 M ammonium sulfate dissolved in EB. The precipitation was carried out for 30 min on ice and subjected to centrifugation at 10⁴ rpm for 20 min in a Sorvall centrifuge. The pellet was resuspended in 1 vol modified EB (10% glycerol, 80 mm β-glycerolphosphate, 7.5 mM MgCl₂, 2 mM EGTA, 10 mM Hepes/NaOH, pH 7.5, 1 mM DTT, 100 mM PMSF) and dialyzed against the same buffer. Aliquots were frozen in liquid nitrogen and stored at −70°C until use. The final protein concentration of the 30% ammonium sulfate fractions of interphase (30% INT) and mitotic extracts (30% MIT) was 8 and 7 mg/ml, respectively.

## Transcription Reactions with Partially Purified Factors

Purification of transcription factors and polymerase from Xenopus oocyte S-150 extracts, as well as transcription reaction conditions, were as described (Keller et al., 1990). Briefly, reactions contained 18 ng of purified TFIIIA (0.47 pmole, a 10-fold molar excess over 5S genes; Millstein et al., 1987), 2 μl of B-block Sepharose-purified TFIIIC (containing 100 fmoles of TFIIIC DNA-binding activity; Keller et al., 1990) and 12.5 μl of a phosphocellulose TFIIIB-RNA polymerase III fraction (Keller et al., 1990) in a final reaction volume of 25 μl. The TFIIIA fraction was ~85% pure; TFIIIC was 2,000–5,000-fold purified over the starting S-150, and the TFIIIB-RNA polymerase III fraction was 20-fold purified over the S-150 fraction (~80-fold purified over oocyte homogenate). Protein fractions were dialyzed against a buffer containing 100 mM KCl, 20 mM Hepes, pH 7.5, 1 mM MgCl₂, 2 mM DTT, and 10% glycerol. Reactions were supplemented with MgCl₂ to 6 mM. 5S gene transcription reactions contained 100 ng of pXls11 and 400 ng of pBR322 carrier DNA. tRNA reactions contained 500 ng of pPC-1. DNA was mixed with factors, polymerase, and NTPs (at 600 μM ATP, CTP, UTP, and 20 μM GTP) followed 30 min later by 5 μl of either modified EB, 30% INT, or 30% MIT interphase and mitotic fractions. Samples were incubated an additional 30 min and the transcription assay was then initiated by adding 10 μCi α-$^{32}$P-GTP. Where DMAP was added, a 300 mM stock solution was first diluted fivefold in modified

EB. The 60 mM DMAP solution was then mixed with the appropriate 30% INT or MIT fraction immediately before addition to the transcription factors and template, so that the final concentration of DMAP in the reaction was 3 mM. Reactions were stopped 120 min after α-$^{32}$P-GTP addition.

## Supercoiling Analysis of Template DNA

To assay the effect of increasing concentrations of carrier DNA on mitotic repression, aliquots were removed from each transcription incubation immediately prior to the addition of α-$^{32}$P-GTP. These aliquots were then mixed with an equal volume of 1% SDS, 20 mM EDTA, 50 mM Tris-Cl, pH 8.0, and 2 mg/ml Proteinase K. The samples were incubated at 37°C for 1 h and then electrophoresed in 1% agarose-TBE gels. Gels were stained with ethidium bromide in TBE and destained in water. DNA was visualized with ultraviolet illumination and photographed. The loss of nucleosomes correlates with changes in supercoiling and can be visualized as distinct band shifts (Simpson et al., 1985).

## Depletion of cdc2 and Related Kinases by pl3-Sepharose

Bacterially expressed pl3$^{suc1}$ was purified and coupled to CNBr-activated Sepharose as described in Dunphy et al. (1988) and Pfaller et al. (1991). pl3- or control-Sepharose 6 M beads were washed with egg lysis buffer containing 5 mg/ml BSA (Gibco-BRL, Inc., Gaithersburg, MD) and then washed again with lysis buffer alone. Interphase or mitotic extracts (200 μl; 1 h after cyclin conversion), generated as described above, were incubated with a 0.5 vol of beads (100 μl) for 15 min at 4°C. Cytosol treated in this manner, either cdc2 depleted or mock depleted, was recovered after removal of beads by centrifugation. Depleted extracts were stored at −70°C until use. Depletion of cdc2 kinase was verified by assaying H1 kinase activity.

## Histone H1 Kinase Assay

Histone H1 kinase activity was assayed as described in Smythe and Newport (1992). Immediately before transcription analysis (i.e., 60′ after cyclin addition), two microliter aliquots were removed from extracts, diluted with an equal volume of EB, and frozen in liquid nitrogen. Upon thawing, the samples were diluted 50-fold in the same buffer. Aliquots of the diluted sample were assayed for H1 kinase activity at a final 200-fold dilution by phosphorylation of calf thymus H1 (Boehringer Mannheim Biochemicals) with γ-$^{32}$P-ATP. H1 kinase activity was quantitated by scintillation counting of phosphorylated H1 bound to phosphocellulose paper (Whatman, P81).

# Results

## A Mitotic Repression of RNA Polymerase III Transcription Can Be Reproduced In Vitro

The eggs of Xenopus laevis, when lysed in the presence of cycloheximide, produce a stable, cell-free, interphase extract. This extract can be separated by centrifugation into clarified cytosol, membrane, and particulate fractions (Lohka and Masui, 1983; Newport, 1987; for review see Smythe and Newport, 1991). The clarified interphase cytosol can in turn be driven into a mitotic state by the addition of bacterially expressed sea urchin cyclin B, which contains a deletion in the amino terminus (Δ13-cyclin; Fig. 1 B; Murray et al., 1989; Solomon et al., 1990). This deletion renders Δ13-cyclin resistant to the normal proteolytic degradation that inactivates MPF at the end of mitosis, causing a stable arrest in mitosis (Murray et al., 1989; Glotzer et al., 1991). It is possible to monitor such conversion of extracts to mitosis by measuring the histone H1 kinase activity of MPF or by observing the formation of mitotic chromosomes from added test nuclei (for review see Smythe and Newport, 1991).

To attempt to observe cell cycle-dependent effects on transcription, interphase cytosol was combined with one of two genes, that for the yeast tRNA$^{leu-3}$ or that for the Xenopus
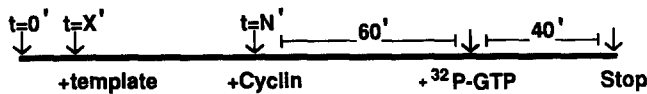
*Figure 1.* Standard protocol for transcription experiments in *Xenopus* egg extracts arrested in either interphase or mitosis. Interphase extracts, prepared as described in Materials and Methods, could be stably driven into mitosis through the addition of bacterially expressed Δ13-cyclin. The time of template ($t = 0 + X'$) and cyclin ($t = 0 + N'$) addition to the extract was varied and always measured from the initiation of the experiment ($t = 0'$). 60 min after buffer or Δ13-cyclin addition, $\alpha$-$^{32}$P-GTP was added and transcription was monitored after a 40-min pulse. At that time, the reaction was stopped and labeled transcripts were quantitated as described in Materials and Methods.



*Figure 2.* Transcription of 5S and tRNA genes is repressed in mitotic extracts. (*A*) Yeast tRNA$^{leu}$ (pPC-1) or *Xenopus* somatic 5S (pXls11) template DNA was added to an interphase egg extract at the times indicated above the figure, as measured from the onset of the experiment. Δ13-cyclin was then added to the indicated samples at the times shown, as measured from $t = 0'$. 1 h after cyclin addition, $\alpha$-$^{32}$P-GTP was added and transcription was monitored for a 40-min pulse, as outlined in Fig. 1. An autoradiograph of labeled transcripts after polyacrylamide gel electrophoresis is shown. Odd numbered lanes show transcripts from extracts converted to mitosis by Δ13-cyclin addition, while even lanes contain transcripts from parallel reactions performed in interphase extracts. Transcription of the yeast tRNA$^{leu}$ template in *Xenopus* extracts generates the primary transcript (*upper band*), and faster migrating products of RNA processing, the most abundant of which is generated by removal of the 5' leader and 3' trailer sequences (Otsuka et al., 1981). The reactions shown in lanes *1-12* contained a tRNA$^{leu}$ template while those in lanes *13* and *14* contained *Xenopus* 5S template. (*B*) Rate of transcription complex formation in interphase cytosol. tRNA template DNA (pPC-1) was added to interphase cytosol at $t = 0'$ and transcription assayed in 15-min pulses starting from the time of template addition. The level of transcription at the end of each pulse was quantitated by electrophoresis of the labeled RNA and densitometric scanning of autoradiographs, as described in Materials and Methods. The average of two separate experiments is shown after normalization for variation in extract transcriptional efficiency. (*C*) Histone H1 Kinase activity of the transcription incubations in *A*. 2-$\mu$l aliquots of extract were removed from each incubation just before $\alpha$-$^{32}$P-GTP addition and immediately frozen in liquid nitrogen. Upon thawing, H1 kinase activity was assayed by phosphorylation of calf thymus histone H1 with $\gamma$-$^{32}$P-ATP, as described in Materials and Methods.

somatic 5S RNA. These genes have been shown to be actively transcribed in *Xenopus* transcription systems (Carrara et al., 1981; Otsuka et al., 1981; Mattoccia et al., 1988; Wolffe and Brown, 1987; for review see Millstein and Gottesfeld, 1989). Experiments were performed with the genes contained in both plasmid and λgt11 vectors. Both vector DNAs assemble into chromatin in interphase extracts; λDNA is further capable of higher-order assembly and mitotic condensation as described in the Introduction. Transcription of the tRNA$^{leu-3}$ gene requires the participation of transcription factors TFIIIC and TFIIIB, which are present in the extract. Transcription of the 5S RNA gene requires, in addition, TFIIIA, which is limiting in *Xenopus* egg extracts (for review see Geiduschek and Tocchini-Valentini, 1988). Purified TFIIIA was therefore preincubated with the 5S template before addition to the extract.

To begin to look for cell cycle effects, template DNA was added to clarified interphase cytosol as outlined in Fig. 1. Buffer or cyclin was then added at $t = 0 + N'$. In buffer-supplemented reactions, the extract remained in interphase. In cyclin-supplemented reactions, the extract was converted to mitosis 45-60 minutes later (as assayed by histone H1 kinase activity). 60 min after cyclin or buffer addition, $\alpha$-$^{32}$P-GTP was added and transcription monitored for a 40-min pulse (Fig. 1). At the end of the pulse the labeled transcripts were isolated, separated by gel electrophoresis, and quantitated as described in Materials and Methods. As expected, in interphase extracts both the yeast tRNA$^{leu-3}$ and the 5S genes were efficiently transcribed (Fig. 2 *A*, lanes 2 and *14*, respectively). In contrast, when template DNA was added to an interphase extract at $t = 0$ min and the extract then converted to mitosis for 60 min, transcription was found to be clearly repressed. This repression was seen both with the tRNA and the 5S templates (Fig. 2 *A*, lanes *1* and *13*, respectively). Quantitation for the experiment in Fig. 2 *A* indicated that a 15-fold repression was observed for transcription of the tRNA gene and 4.5-fold repression for the 5S gene. These represent typical values for the mitotic repression we observe. Repression was seen whether the tRNA gene was present in a plasmid vector (Fig. 2) or a λgt11 vector (see Fig. 4 *C*, compare lane *1* to lane *6*). In all reactions where cyclin had been added, conversion to a mitotic state was confirmed by showing the induction of H1 kinase activity (Fig. 2 *C*, odd-numbered lanes). Significantly, when the general protein kinase inhibitor dimethylaminopurine (DMAP) was added at the time of cyclin addition, mitotic repression
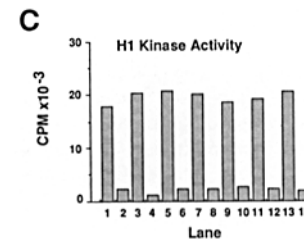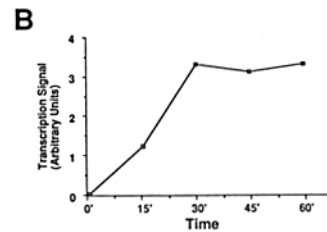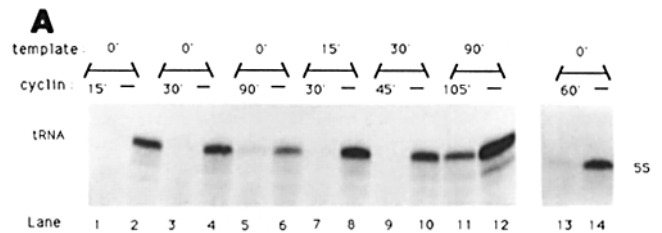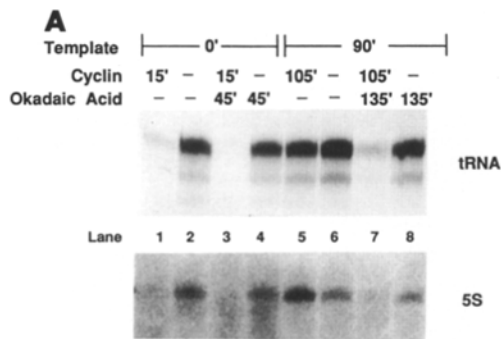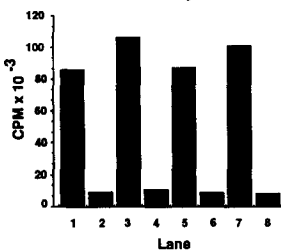
was abolished. DMAP had no effect on interphase transcription (data not shown; however, a similar result is seen in Fig. 7 *A*, lanes *4* and *6*). From these results, we conclude that we have been able to recreate a mitotic repression of RNA polymerase III transcription in vitro and that this repression requires the activity of a mitotic protein kinase, since it can be prevented by the kinase inhibitor DMAP.

When the time of template and cyclin addition was varied, results identical to those described above were seen: transcription was very active in interphase extracts, while it was strongly repressed in mitotically converted extracts (Fig. 2 *A*, lanes *1-10* and *13-14*). An interesting exception to the ability of mitotic extracts to repress transcription was found

**A**



| Template | |—— 0' ——| |—— 90' ——| |
| Cyclin | 15' | – | 15' | – | 105' | – | 105' | – |
| Okadaic Acid | – | – | 45' | 45' | – | – | 135' | 135' |

Lane 1 2 3 4 5 6 7 8

tRNA

5S

**B**

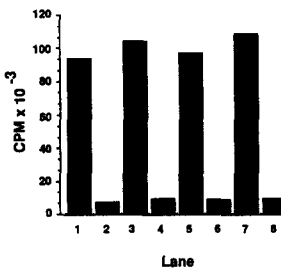H1 Kinase Activity - tRNA



H1 Kinase Activity - 5S



*Figure 3.* Effect of okadaic acid on transcription in interphase and mitotic extracts. (*A*) The upper panel shows tRNA (pPC-1) transcripts and the lower panel 5S RNA (pXls) transcripts. As indicated above the figure, template DNA was added to interphase cytosol at the onset of the experiment (0') or after a 90-min incubation of extract at room temperature. Cyclin was added to the samples fifteen minutes after the addition of template DNA. Okadaic acid (2-μm final) was then added to the indicated samples 30 min after the addition of cyclin. 60 min after the addition of cyclin (or buffer) α-$^{32}$P-GTP was added and transcription assayed for a 40-min pulse. Even-numbered lanes show transcripts from interphase incubations, odd-numbered lanes show products from mitotically converted samples. Okadaic acid clearly restored mitotic repression to extracts incubated for lengthy times (compare lane 7 to lane 5); a nonfunctional methylester analogue of okadaic acid could not restore repression (data not shown). (*B*) H1 kinase activity of the transcription incubations in *A* was uniformly high in the cyclin-added lanes and uniformly low in the buffer-added lanes.

when the interphase extract was preincubated for long periods of time at room temperature prior to template and cyclin addition. In this case, repression was substantially reduced (Fig. 2 *A*, lane *11*) or abolished (Fig. 3 *A*, lanes *5*), despite the induction of a normal level of H1 kinase activity (Fig. 2 *C*, lane *11*; Fig. 3, lanes *5*). Repression could be restored in such an extract, however, if okadaic acid, an inhibitor of the protein phosphatases type 1 and 2A, was added (Fig. 3 *A*, lane *7*; compare to lane *5*). Since okadaic acid had little effect on interphase transcription (Fig. 3 *A*, lanes *4* and *8*), inhibition of a protein phosphatase activity in interphase extracts is not sufficient to induce repression. These results reiterate the importance of an active mitotic protein kinase in mediating transcription repression at mitosis. The kinase may become attenuated when the extract is subjected to long preincubation, such that an okadaic acid–sensitive phosphatase can now antagonize repression. The regulatory interactions of the phosphatases and kinases which control mitotic

transcriptional repression are addressed further in the last section of the Results.

A more immediate goal was to investigate the mechanism of the mitotic repression that we observe. We first asked whether the repression is acting on preformed transcription complexes, as would be expected for in vivo mitotic repression. Pulse label experiments showed that within 30 min of template addition to interphase extracts the rate of α-$^{32}$P-GTP incorporation became constant (Fig. 2 *B*), indicating that complex formation was complete within that time. We could thus conclude that the repression observed in extracts incubated with template DNA for at least 30 min before mitotic conversion, as is done in all our experiments, must reflect inhibition of transcription from preformed transcription complexes (Fig. 2 *A*, lanes *3*, *5*, and *13*), analogous to the mitotic repression of active genes in vivo.

## VM-26, an Inhibitor of Chromosome Condensation, Does Not Prevent Mitotic Repression of Transcription In Vitro

Having established an in vitro system that mimics mitotic repression of preformed pol III transcription complexes, a possible role of chromosome condensation in this repression was addressed. Topoisomerase II (topo II) activity has been clearly shown to be required for normal mitotic chromosome condensation both by inhibition and immunodepletion of *Xenopus* extracts (Newport and Spann, 1987; Adachi et al., 1991) and by temperature-sensitive mutants of topo II in *Schizosaccharomyces pombe* (Uemura et al., 1987). A specific inhibitor of topo II, the epipodophyllotoxin VM-26, prevents topo II activity by allowing the enzyme to make a double-stranded cut in DNA, but arresting topo II while bound to unligated DNA ends (Chen et al., 1984; Liu, 1989). Inactivation can be easily assessed with circular plasmid templates: the supercoiling induced by nucleosome assembly is released when the plasmid is linearized by topo II plus VM26. In *Xenopus* extracts, VM26 blocks mitotic chromosome formation (Newport and Spann, 1987), as well as the normal mitotic condensation of chromatin assembled from exogenously added DNA (Hirano and Mitchison, 1991).

To analyze a role for condensation in our observed repression, VM-26 was added to interphase extracts immediately before the addition of template DNA. In interphase extracts, VM-26 slightly enhanced the transcription of 5S and tRNA genes (maximum twofold; Fig. 4 *A*, even-numbered lanes) (Gottesfeld, 1986; Razik et al., 1989; Wolffe et al., 1987). Addition of VM-26 did not interfere with the conversion of these extracts to mitosis by cyclin addition, as indicated by the induction of H1 kinase activity (Fig. 4 *B*, odd numbered samples). Significantly, VM-26 did not prevent the mitotic repression of transcription from a plasmid template (Fig. 4 *A*, odd numbered lanes). VM-26 also did not prevent mitotic transcription repression when the tRNA gene was contained in a λgt11 vector (Fig. 4 *C*). Lambda DNA templates have been shown to assemble into chromatin and to condense tightly upon conversion to mitosis, the latter process being prevented by VM-26 (Hirano and Mitchison, 1991). Having found that concentrations of VM-26 ≥250 μM were sufficient to inactivate topo II in the interphase cytosol, as assessed by the arrest of the plasmid template in a linear form (Fig. 5), our data indicate that the chromatin condensation
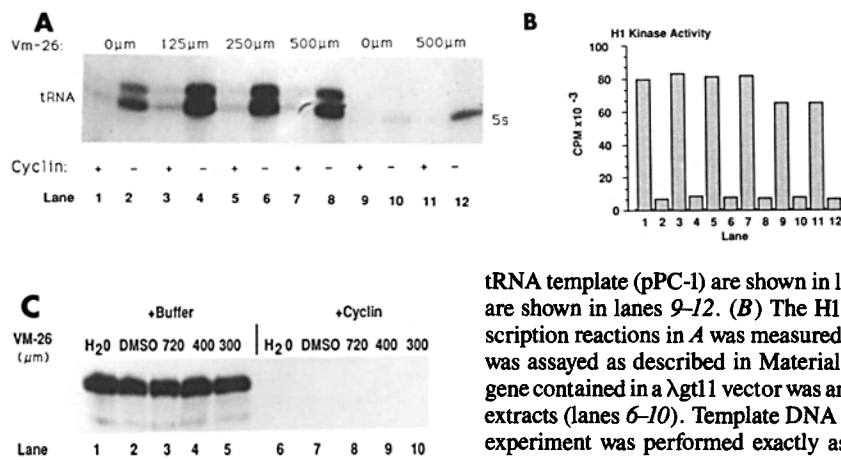
A
Vm-26: 0μm 125μm 250μm 500μm 0μm 500μm
tRNA
5s
Cyclin: + – + – + – + – + – + –
Lane 1 2 3 4 5 6 7 8 9 10 11 12

B
H1 Kinase Activity
CPM x10⁻³
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12
Lane

C
VM-26 (μm)        +Buffer                    +Cyclin
        H₂0 DMSO 720 400 300 | H₂0 DMSO 720 400 300
Lane      1   2   3   4   5    6   7   8   9   10

*Figure 4.* VM26, an inhibitor of normal chromatin condensation, does not prevent mitotic transcriptional repression in vitro. (*A*) VM-26 or the solvent DMSO were added to interphase extracts immediately before template DNA addition at the beginning of the experiment. Cyclin was added 60 min later. After a further 60-min incubation, $\alpha$-$^{32}$P-GTP was added and transcription assayed for a 40-min pulse as described in Fig. 1. Transcripts from tRNA template (pPC-1) are shown in lanes *1–8*, while those from the 5S template (pXls11) are shown in lanes *9–12*. (*B*) The H1 kinase activity of the interphase and mitotic transcription reactions in *A* was measured. Kinase activity in 2-$\mu$l aliquots of each incubation was assayed as described in Materials and Methods. (*C*) Transcription of the tRNA$^{leu-3}$ gene contained in a λgt11 vector was analyzed in interphase (lanes *1–5*) or cyclin-converted extracts (lanes *6–10*). Template DNA was added to a final concentration of 24 ng/μl. The experiment was performed exactly as in *A*.

mediated by topo II does not play a major role in the mitotic repression of transcription that we observe in vitro. A normal level of repression is observed in the absence of full condensation.

### Neither Chromatin Structure Nor a Titratable Chromatin-binding Factor Are Required for the Mitotic Repression of Transcription In Vitro

Since nucleosomes readily form when template DNA is added to *Xenopus* extracts, (Laskey et al., 1977), it was possible that either a modification of nucleosome structure or the activation of a repressive chromatin-binding protein in the extract could be mediating the observed repression of transcription during mitosis. One can prevent the assembly of nucleosomes onto a transcription template if a *Xenopus* transcription extract is first preincubated with a large concentration of non-specific DNA (Laskey et al., 1977; Wolffe and Brown, 1987; Almouzni et al., 1990). During this preincubation period, both histone proteins and chromatin-binding proteins bind to the non-specific DNA and are thus



Vm-26    | 0μm | 10 μm | 50 μm | 125 μm | 250 μm |
-Ir,II
-III
-I
time (minutes)   30,120 | 30,120 | 30,120 | 30,120 | 30,120

*Figure 5.* Template DNA in egg extracts: effect of VM-26. tRNA plasmid DNA (pPC-1) was added to egg extracts containing the indicated concentrations of VM-26 at $t = 0$ min. Δ13-cyclin was added 60 min after template DNA. Aliquots were removed for template analysis after 30- and 120-min incubations with cyclin. Samples were treated with SDS, EDTA, and Proteinase K as described in Materials and Methods, and electrophoresed on a 1% agarose-TBE gel to determine the level of supercoiling. DNA forms I, Ir, II, and III indicate supercoiled, relaxed circular, nicked circular, and linear templates, respectively. The last lane contains the untreated supercoiled template DNA used in these experiments.

unavailable to form nucleosomes on a DNA template when it is later added to the transcription reaction.

To test the requirement for nucleosome formation or chromatin binding of a general repressive factor in mitotic repression, we preincubated interphase extracts with increasing quantities of non-specific DNA (10-kb plasmid, pMB258). Chromatin formation on subsequently added template DNA was blocked by preincubation with non-specific DNA concentrations as low as 83 ng/ul, as indicated by the loss of normal supercoiling conferred by nucleosome deposition (Fig. 6 *B*; Simpson et al., 1985). In the absence of non-specific DNA, mitotic repression was observed as usual (Fig. 6 *A*, lane *1*). The presence of intermediate non-specific DNA concentrations resulted in an enhancement of transcription of the template in the interphase extract (Fig. 6 *A*, lanes *4* and *6*), due to the increased efficiency of transcription complex assembly when nucleosomes are absent (Gottesfeld and Blommer, 1982; Wolffe and Brown, 1987; Razik et al., 1989; Felts et al., 1990; Almouzni et al., 1990, 1991). Very high non-specific DNA concentrations led to a relative decrease in transcription in interphase extracts as compared to intermediate DNA concentrations (Fig. 6 *A*, lane *10*). This is due to the progressive titration of promoter-specific transcription factors (Wolffe and Brown, 1987; Almouzni et al., 1990). The low level of mitotic transcription, sometimes observed in our experiments (Fig. 6 *A*, lane *1*) and by implication in vivo (Gazit et al., 1982; Kerem et al., 1984), responded in the same manner to increasing DNA concentrations (Fig. 6 *A*, lanes *3*, *5*, *7*, *9*). Significantly, however, at all nonspecific DNA concentrations, transcription in mitotic extracts remained repressed in the same relative ratio when compared to interphase extracts (Fig. 6 *A*). Even at the highest concentration of DNA (330 ng/μl), the ratio of mitotic repression of transcription observed was identical to that found in the absence of non-specific DNA (Fig. 6 *A*, lanes *9* and *10* vs. lanes *1* and *2*). Similar results have been obtained with the 5S gene as template or when using λDNA as the non-specific competitor DNA (data not shown). We conclude from the normal ratio of repression observed in the absence of chromatin, that neither nucleosome formation, nor the binding of a possible general repressive factor such as histone H1 to chromatin is required to mediate the mitotic repression of transcription in vitro.
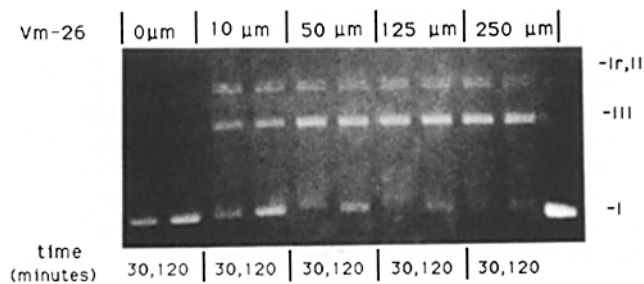
**A** Lane 1 2 3 4 5 6 7 8 9 10

Competitor DNA ng/μl | 0 | 42 | 83 | 165 | 330 |

Cyclin: + − + − + − + − + −

**B**

Competitor DNA (ng/μl) — 0 42 83 165 330

- Ir,II

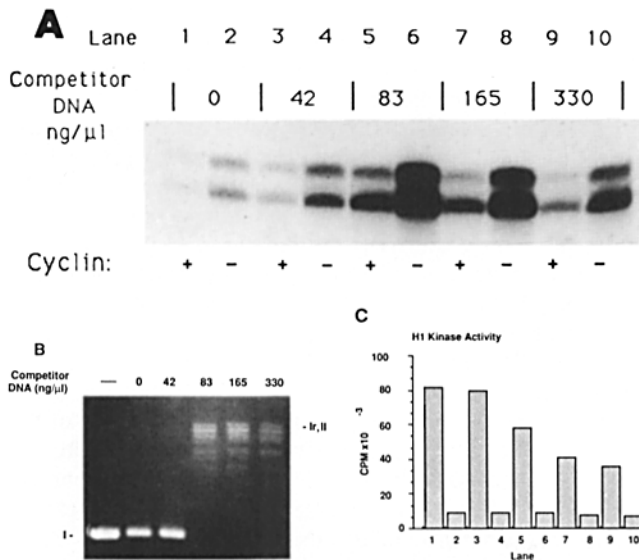I-

**C**

H1 Kinase Activity

*Figure 6.* Inhibition of chromatin formation in egg extracts has no effect on the mitotic repression of transcription in vitro. (*A*) Non-specific DNA (plasmid pBMB258, 10 kb) was added to interphase extracts at *t* = 0' to the final concentrations indicated in the figure. At *t* = 120', tRNA template DNA (pPC-1) was added to all samples, followed by Δ13-cyclin addition at *t* = 180' where indicated. A 40-min transcription pulse was initiated at *t* = 240' and stopped at 280'. It should be noted that okadaic acid (2-μm final) was added to all incubations at *t* = 210' to inhibit a decay in the repressive activity seen with long preincubations at room temperature (as in Fig. 3). A long preincubation with non-specific DNA was required for this experiment to titrate chromatin formation before template DNA addition. An autoradiograph of labeled transcripts is shown. (*B*) The topological forms of the template DNA in *A* are shown. Immediately before transcription analysis (*t* = 240'), aliquots from each incubation were removed and processed for analysis of the non-specific DNA as described in Materials and Methods. DNA was electrophoresed on a 1% agarose-TBE gel. The first lane contains the untreated supercoiled template DNA (form I) added to the extract. The positions of relaxed (form Ir) and nicked circular (form II) DNA are indicated. The multiple bands in lanes 83, 165, and 300 are typical of protein-free relaxed DNA. (The 10-kb non-specific DNA plasmid migrated in the upper portion of the gel and showed parallel inhibition of supercoiling. This portion of the gel was cut off and is not shown.) (*C*) H1 kinase measurements for the incubations shown in *A*. Aliquots were assayed as described in Materials and Methods. The twofold decrease in H1 activity at the highest concentration may be due to the affinity of *cdc2* for DNA (Fotedar and Roberts, 1991).

## Mitotic Repression of Transcription Occurs with Partially Purified Transcription Factors and Polymerase

An alternative mechanism for the observed inhibition of transcription might be a mitotic phosphorylation of RNA polymerase III itself or associated transcription factors. To test this mechanism, a protein fraction enriched for the mitotic *cdc2* kinase was prepared from a mitotic extract by precipitation with 30% ammonium sulfate (Wu and Gerhart, 1980; Dunphy and Newport, 1988; Pfaller et al., 1991). An identical protein fraction was prepared from interphase extracts as a control. Each of these fractions contains approximately 10% of the total protein of the clarified *Xenopus* egg
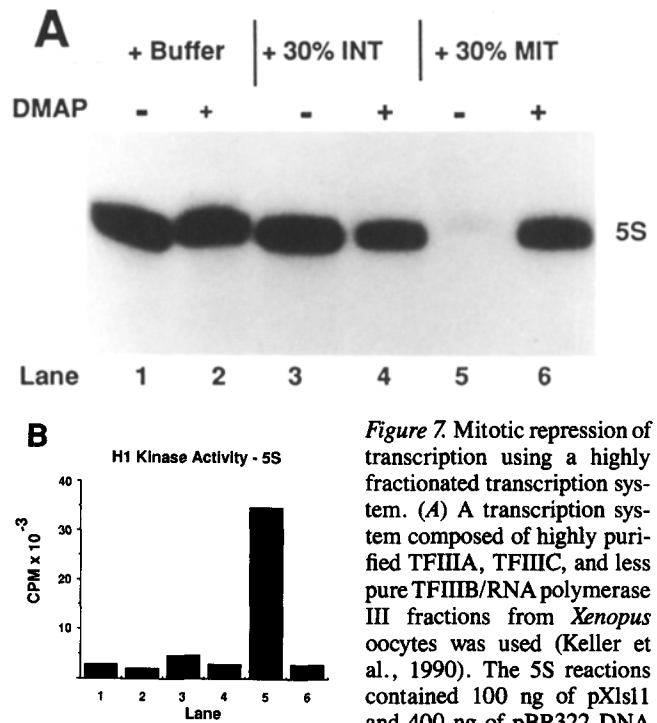


**A** + Buffer | + 30% INT | + 30% MIT

DMAP − + − + − +

5S

Lane 1 2 3 4 5 6

**B**

H1 Kinase Activity - 5S

*Figure 7.* Mitotic repression of transcription using a highly fractionated transcription system. (*A*) A transcription system composed of highly purified TFIIIA, TFIIIC, and less pure TFIIIB/RNA polymerase III fractions from *Xenopus* oocytes was used (Keller et al., 1990). The 5S reactions contained 100 ng of pXls11 and 400 ng of pBR322 DNA as carrier. The DNA was mixed with the above transcription factors and NTPs and incubated for 30 min to allow transcription complexes to form. 5 μl of a 30% ammonium sulfate fraction of either the interphase (30% INT) or mitotic extract (30% MIT) were then added to the indicated samples. Reactions were incubated an additional 30 min and then assayed for transcription by the addition of α-32P-GTP. Reactions were stopped 120 min later and processed and electrophoresed as described previously (Keller et al., 1990). In lanes 2, 4, and 6, DMAP was added (3 mM final) simultaneously with the addition of the egg extract ammonium sulfate fractions. (*B*) Histone H1 kinase activity of the reactions depicted in *A* was assayed as in previous experiments.

cytosol. Alone, these fractions were unable to transcribe added template DNA (5S or tRNA) and had no nucleosome forming ability (data not shown). The fractions were assayed for an effect on a highly simplified pol III transcription system composed of partially purified *Xenopus* RNA polymerase III and pol III transcription factors (Keller et al., 1990). Since the simplified system was optimized for 5S gene transcription, somatic 5S template DNA, rather that the tRNA template, was incubated with the purified transcription components for 30 min to allow transcription complexes to form. The reactions were then combined with aliquots of either the *cdc2* kinase-enriched fraction of a mitotic extract (30% MIT) or the equivalent fraction of an interphase extract (30% INT). With the interphase fraction (used as a control for the addition of a subset of egg proteins to the more purified transcription reaction), activated 5S DNA template was transcribed as efficiently as in the original optimized reaction (Fig. 7 *A*, lane *3*, compare to lane *1*). In contrast, the 5S transcription reaction to which the mitotic kinase-enriched fraction (30% MIT) was added showed strong mitotic repression of transcription (Fig. 7 *A*, lane *5*, compare to lanes *1* and *3*). Strong repression by the mitotic fraction was also observed for all other RNA polymerase III tem-
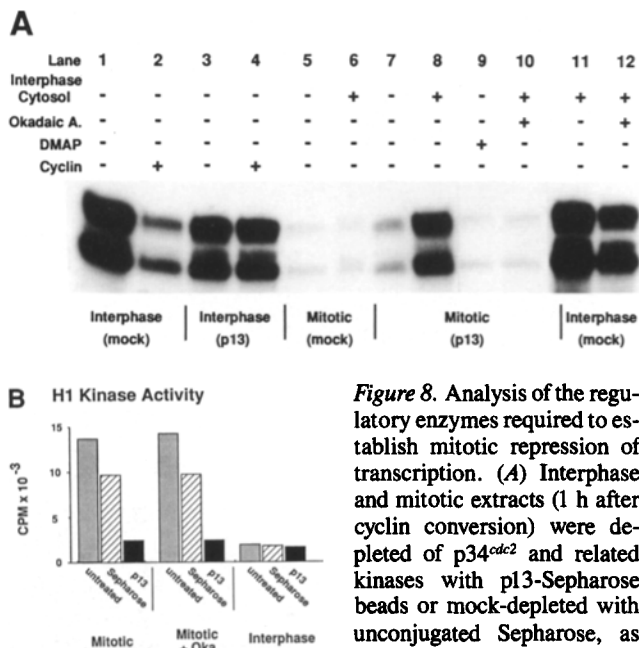
A

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Interphase Cytosol | - | - | - | - | - | + | - | + | - | + | + | + |
| Okadaic A. | - | - | - | - | - | - | - | - | - | + | - | + |
| DMAP | - | - | - | - | - | - | - | - | + | - | - | - |
| Cyclin | - | + | - | + | - | - | - | - | - | - | - | - |

Interphase (mock) | Interphase (p13) | Mitotic (mock) | Mitotic (p13) | Interphase (mock)

B  H1 Kinase Activity

*Figure 8.* Analysis of the regulatory enzymes required to establish mitotic repression of transcription. (*A*) Interphase and mitotic extracts (1 h after cyclin conversion) were depleted of p34$^{cdc2}$ and related kinases with p13-Sepharose beads or mock-depleted with unconjugated Sepharose, as described in the Materials and Methods. At the initiation of the experiment ($t = 0'$), 30-$\mu$l aliquots of such extracts were combined with the reagents shown above the figure. The concentrations of added reagents were 2 mM okadaic acid, 3 mM DMAP, or one-tenth volume cyclin (lanes 2 and 4) or XB buffer (lanes 1 and 3). In reactions composed of a mixture of extracts (lanes 6, 8, and 10), 20 $\mu$l of the mitotic extract (mock or cdc2 depleted) was combined with 15 $\mu$l of interphase extract before adding template DNA. The capacity of such extracts to mediate mitotic repression of transcription was then analyzed by adding tRNA template (pPC-1) at $t = 10'$. A transcriptional pulse was initiated at $t = 100'$ and terminated at $t = 140'$. (*B*) The efficiency of cdc2 kinase-depletion in p13- and mock-depleted extracts was assayed by measuring the level of H1 kinase activity before and after bead treatment as described in Materials and Methods.

plates tested, including the genes for *Xenopus* oocyte 5S RNA, tRNA$^{met}$, the *Xenopus* satellite I transcript, and yeast tRNA$^{leu-3}$ (data not shown). Significantly, mitotic repression of transcription was not observed if the kinase inhibitor DMAP was present (Fig. 7 *A*, lane 6). Thus, the data indicate that a component of the simplified transcription system, either RNA polymerase III or an accessory protein, is inhibited by phosphorylation with a mitotic kinase. Mitotic repression can be abolished when the mitotic kinase is blocked with DMAP.

## Mitotic Repression of Transcription Can Occur in Extracts Depleted of cdc2 Kinase

The results presented above indicate that the inhibition of transcription at mitosis requires phosphorylation by a mitotic protein kinase. This phosphorylation could be mediated directly by the active form of cdc2 kinase (MPF) or, alternately, by a secondary kinase activated at mitosis by cdc2 kinase. To determine first whether cdc2 kinase is essential for maintaining transcriptional repression once it is established, the following experiment was done: an interphase extract was converted to mitosis by cyclin addition. 60 min after cyclin addition when the extract was fully converted to mitosis (Fig. 8 *B*, column *1*), cdc2 and related kinases were depleted

using p13-Sepharose beads (Brizuela et al., 1987; Dunphy et al., 1988; Fang and Newport, 1991; Pfaller et al., 1991). Effective depletion of cdc2 kinase (and related kinases) by the p13-beads was indicated by the reduction in H1 kinase activity to interphase levels (Fig. 8 *B*, third bar). Upon addition of tRNA template, the cdc2-depleted mitotic extract strongly repressed transcription (Fig. 8 *A*, lane 7; compare to lane 3). These data indicate that cdc2 is required to activate the mitotic repression of transcription, but not to maintain the system that mediates transcription repression. When the kinase inhibitor DMAP was added to the cdc2-depleted mitotic extract, repression of transcription was still observed (Fig. 8 *A*, lane 9). This result suggests that the transcriptional proteins(s) involved in transcriptional repression are phosphorylated by the time of cdc2 removal and remain in the repressed state after its removal. The results also indicate that a phosphatase, which could reverse the phosphorylation, is not active in the cdc2-depleted mitotic extract.

These experiments do not distinguish between a phosphorylation of the transcription machinery by cdc2 kinase or phosphorylation by a secondary downstream kinase activated by cdc2 kinase. To address this point, we asked whether a cdc2-depleted mitotic extract could repress an aliquot of interphase extract. We added cdc2-depleted extract to the interphase extract (at a ratio of 1.3:1). Upon addition of template, transcription was now observed at high levels, indicating that the interphase state was dominant (Fig. 8 *A*, lane 8). However, if the phosphatase inhibitor okadaic acid was included in the interphase extract, then when the cdc2-depleted mitotic extract was added a normal mitotic repression of transcription was observed (10-fold reduction; Fig. 8 *A*, lane 10). (An interphase extract alone treated with okadaic acid transcribed at the normally high rate [Fig. 8 *A*, lane 12; see also Fig. 3, lanes 4 and 8]). These results indicate that there is a phosphatase present in interphase extracts which must be inactivated in these mixing experiments to observe mitotic repression. Once this phosphatase is inactivated by okadaic acid, however, a kinase activity in the cdc2-depleted mitotic extract is fully capable of transcription repression (Fig. 8 *A*, lane 10), even though the mitotic extract has been depleted of cdc2 kinase (Fig. 8 *B*, column 6). Together these results indicate that a secondary kinase, activated by cdc2 kinase, can mediate mitotic repression, since this repressive activity is present only in mitotic extracts and remains in an active form after depletion of cdc2 kinase. The results also suggest that one role of cdc2 kinase is the inhibition of an interphase phosphatase during mitosis, since a cdc2-depleted mitotic extract is unable to inactivate interphase phosphatase activity (Fig. 8 *A*, lane 8).

## Discussion

When eukaryotic cells undergo mitosis, DNA becomes detached from the nuclear matrix, undergoes condensation to form mitotic chromosomes, and the nucleus is broken down. During this process many nuclear proteins become phosphorylated, including proteins fundamental to chromatin structure, such as topoisomerase II, histone H1, and histone H3, as well as factors specific for the transcription of individual genes (Heck et al., 1989; Bradbury et al., 1974; Bradbury, 1992; Roberts et al., 1991). Concomitant with these dramatic mitotic changes, transcription becomes in-

hibited. The mechanisms generating the global repression of transcription at mitosis have been the subject of considerable speculation, but to date an in vitro system mimicking mitotic repression of transcription has been lacking. In this report we have developed a system capable of achieving a mitotic repression of RNA polymerase III transcription in vitro. As observed in vivo, transcription appears to be inhibited from preformed transcription complexes when interphase extracts are converted to mitosis. Thus these extracts represent a powerful system for potential analysis of the biochemical and structural mechanisms leading to transcriptional silencing during mitosis.

It has been hypothesized that the transcriptional repression observed during mitosis is mediated by mitotic chromosome condensation (Johnson and Holland, 1965; Lewis et al., 1984). In an extension of this model, it has been speculated, based both on increased phosphorylation and extractability of Oct-1 from mitotic HeLa cells (Segil et al., 1991), as well as on potential p34$^{cdc2}$ kinase sites found in RNA polymerase II (Cisek and Corden, 1989), that a putative phosphorylation of transcription factors at mitosis could cause the loss of the factors, and thus would *allow* the condensation of chromosome and subsequent prevention of transcription (Moreno and Nurse, 1990). Since lambda DNA templates assemble into chromatin when added to interphase extracts and undergo mitotic condensation upon conversion of the extract to mitosis (Newport and Spann, 1987; Hirano and Mitchison, 1991), the role of condensation in the repression we observe could be assessed. VM-26 by blocking topo II blocks both mitotic chromosome condensation and the most highly condensed level of mitotic condensation of chromatin assembled from naked DNA (Newport and Spann, 1987; Adachi et al., 1991; Hirano and Mitchison, 1991). In the presence of VM-26, we found that normal transcriptional repression occurred with both plasmid and λDNA templates, indicating that full chromosome condensation is not a requirement for the mitotic repression of transcription that we observe in vitro. A 75–90% repression of transcription (four- to tenfold) is seen in the absence of the condensation. A possible argument could be made for an involvement of topo I–mediated chromatin condensation in repression. This, however, cannot be a major source of repression, since full repression occurs in the absence of any chromatin structure (Figs. 6 and 7). The conclusion which must again be stressed is that the same level of repression is always observed whether the template is able to condense (Fig. 2), is limited in its condensation but still packaged into nucleosomes (Fig. 4), or is lacking any nucleosomes (Figs. 6 and 7). It seems likely, however, that condensation does play a role in repression in vivo, perhaps producing a further order-of-magnitude repression, for example, from 90 to 99%. In this light, the major role of chromosome condensation at mitosis may be one of providing an additional and powerful fidelity to the overall system of transcriptional repression.

Modification of nucleosome structure or the binding to chromatin of a general repressive factor have also been proposed to mediate transcriptional repression at mitosis. Chromatin formation in *Xenopus* cytosol can be abolished by titrating the endogenous pool of histones with sufficient quantities of non-specific DNA (Laskey et al., 1977; Almouzni et al., 1990). Upon preincubation of extracts with increasing amounts of non-specific DNA before the addition
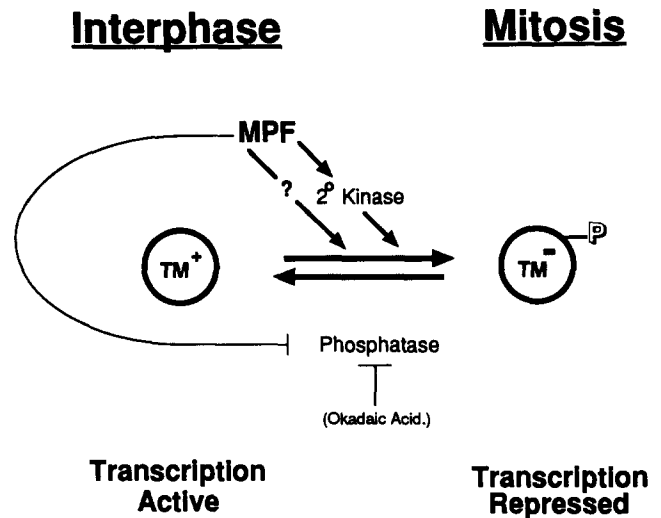


## Interphase                    Mitosis

Transcription Active                    Transcription Repressed

*Figure 9.* Model for the regulatory steps required to mediate mitotic repression of transcription. In this model, repression of transcription requires phosphorylation by a mitotic kinase of a component of the transcriptional machinery proper or a specific accessory factor which interacts with the transcriptional machinery. MPF (*cdc2* kinase) initiates repression. Action via a secondary kinase is suggested by the finding that, upon removal of MPF, a mitotic kinase activity remains and has a dominant effect on any added interphase transcriptional machinery, provided that phosphatase activity in the interphase extract is inhibited with okadaic acid. In vivo at mitosis and in undepleted mitotic extracts, the phosphatase activity may be inactivated at mitosis by the action of MPF. It remains to be determined whether in non-depleted circumstances MPF itself can also phosphorylate the target required for transcriptional repression. *TM*, components of the transcriptional machinery.

of template DNA, we found that the ratio of transcription between mitotic and interphase samples remained constant. This proved true despite the addition of concentrations of DNA high enough to abolish chromatin formation (Fig. 6 *B*; Wolffe and Brown, 1987; Almouzni et al., 1990). These data strongly indicate that neither nucleosome structure nor binding of a general titratable repressive protein, such as phosphorylated H1 or H3, are significant components of the mitotic repression of RNA polymerase III transcription we observe in vitro.

Results presented here instead point toward a direct phosphorylation and inactivation of the transcriptional machinery at mitosis, as outlined in the model in Fig. 9. The data suggest that the target of phosphorylation is either a component of the transcription complex, RNA polymerase itself, or alternatively, an accessory factor which interacts with the transcription complex in a specific manner. This conclusion is based on the findings that: (*a*) elimination of chromatin structure does not reduce the bulk of the mitotic repression observed, as analyzed by several means; and (*b*) a 30% ammonium sulfate fraction of a mitotic extract represses transcription even when added to a highly simplified transcription reaction. In complete cytosol and in this more simplified system, repression is dependent on a mitotic protein kinase and is abolished by the protein kinase inhibitor DMAP. Although it is most probable that the mitotic kinase in the 30% MIT fraction phosphorylates the purified transcriptional machinery, there is a possibility that an accessory transcrip-

tional factor in the 30% MIT fraction itself has been phosphorylated. We think this unlikely since template is fully saturated with the purified transcription machinery in this experiment. In combination, then, the data indicate that the mitotic repression of transcription requires direct phosphorylation of the transcriptional machinery and, furthermore, that this phosphorylation is sufficient to produce the full repression we observe. Indeed, equivalent repression is seen in our experiments whether or not chromatin is formed (Figs. 6 and 7).

Potential transcription factor targets of phosphorylation are those factors used for both 5S and tRNA transcription in the simplified transcription reaction, i.e., the factors TFIIIB, TFIIIC, and RNA polymerase III (Geiduschek and Tocchini-Valentini, 1988). More than one target factor is of course possible. Interestingly, it has been recently reported that the TATA-binding protein of the RNA polymerase II transcription factor TFIID is required for transcription of RNA polymerase III genes in a HeLa cell-free system (White et al., 1992). Recent reports also demonstrate that this TATA-binding protein (TBP) is a common component of RNA polymerase, I, II, and III transcription machinery (Comai et al., 1992; Cormack and Struhl, 1992; Schultz et al., 1992). A TATA-binding protein is present in the TFIIIB-RNA polymerase fraction used in our experiments (J. Gottesfeld, unpublished results). Mitotic phosphorylation of TBP could, thus, potentially serve not only as the target for the repression we observe, but as a general and efficient mechanism for repression of RNA polymerase I, II, and III transcription at mitosis.

To repress transcription by the different RNA polymerases during mitosis, one might predict that there are both common and distinct mechanisms utilized. For example, Shermoen and O'Farrell (1991) have found in vivo that premature termination of elongating RNA polymerase II transcripts is a component of the mitotic repression of the large 77-kb *Ubx* transcript of *Drosophila*. One might speculate that the longer elaborately controlled RNA polymerase II transcripts would be regulated at mitosis by multiple means, i.e., the destabilization of existing elongating transcripts (Segil, 1991; Shermoen and O'Farrell, 1991), as well as by the prevention of new initiation events through the phosphorylation and removal of transcription factors. RNA polymerase III templates, on the other hand, with their relatively simple promoters and short transcripts would likely use the most basic mechanism for mitotic repression of transcription, inhibition of transcription initiation, as the results presented here indicate. A recent report of successful RNA polymerase II transcription in *Xenopus* egg extracts (Tayoda and Wolffe, 1992) should now permit further elucidation of the distinct, as well as the common, mechanisms mediating repression of pol II and pol III transcription during mitosis.

Having demonstrated kinase-dependent repression of transcription at mitosis, it was of further interest to examine the identity of the mitotic kinase, i.e., whether it is the known global initiator of mitosis, cdc2 kinase, or a secondary kinase activated by cdc2 kinase. We found that once cdc2 kinase had fully converted an extract to a mitotic state, the removal of the cdc2 kinase had little effect: repression of transcription from later added template still occurred. Thus, cdc2 kinase is not required for maintenance of the transcription-repressed state. Repression could even be maintained in

such extracts if all kinases, even a potential secondary kinase, were inhibited with DMAP (Fig. 8 A, lane 9). Presumably, this indicates that the transcriptional machinery was already phosphorylated by the time of cdc2 removal, and that there was no phosphatase activity in the mitotic extracts capable of reversing the repression.

To discern whether a secondary kinase plays a role in establishing transcriptional repression, we mixed a cdc2-depleted extract with an interphase extract and found that it could not convert the interphase transcriptional machinery to a mitotically repressed state. However, if interphase phosphatases (type 1, 2a, and 2b) were inhibited with okadaic acid before mixing, the cdc2-depleted mitotic extract was fully capable of converting the interphase extract to a repressed state (Fig. 8 A, lane 10). This observation provides evidence that there is a secondary kinase present in the cdc2-depleted extract that is activated by cdc2 kinase before removal of cdc2. This secondary kinase can then phosphorylate the transcriptional machinery present in the interphase extract, as long as interphase phosphatases are inactivated.

Although it appears that a secondary kinase can mediate mitotic repression of transcription, this does not rule out the possibility that cdc2 kinase itself in an undepleted mitotic extract also contributes to a direct phosphorylation of the transcriptional machinery, a possibility which will be interesting to assess. At present, we do not know the identity of the secondary protein kinase, although efforts are underway to purify it (C. Smythe, personal communication). The cAMP-dependent protein kinase (PKA) has been implicated in the phosphorylation of transcription factor Oct-1 (Segil et al., 1991); however, the addition of the PKA inhibitor peptide, PKI, had no effect on the repression we observe (P. Hartl and C. Smythe, unpublished observations). Future identification of this kinase will help in the refinement of the model presented in Fig. 9.

In addition to being involved in general mitotic repression, phosphorylation of the transcriptional machinery would be predicted to affect certain specific genes. For example, a number of genes are expressed at only one phase of the cell cycle, and are inactive at other phases. Mitotic phosphorylation and removal of the specific transcriptional activators controlling these genes may indeed be part of a mechanism to reset the transcriptional clock at the M/G1 transition each cell cycle. This resetting would help to ensure the correct temporal control of such genes during the cell cycle. The transcriptional activator Oct-1, required for S phase–specific expression of histone H2b gene in HeLa cells (Fletcher et al., 1987), is phosphorylated during mitosis and apparently removed from the chromatin (Segil et al., 1991), consistent with such a resetting scenario. An extension of this mechanism could also function during development. It has been speculated that the programming of genes to be active occurs by the binding of specific transcriptional factors at the time of DNA replication (for review see Brown, 1984; Weintraub, 1985; Wolffe, 1991). To accomplish the opposite effect, i.e., the turning off of genes during development, we propose that mitotic phosphorylation could remove certain stage- or tissue-specific transcriptional activators from the DNA. Subsequent dilution during cell division, without new synthesis, would prevent efficient factor binding and thereby mediate a change in gene expression.

In summary, we have established a cell-free system in

which mitotic repression of RNA polymerase III transcription can be investigated. Neither normal mitotic chromosome condensation nor the presence of nucleosome structure are required for the mitotic repression of transcription that we observe. It also appears unlikely that any repressive chromatin-binding factor activated at mitosis or the presence of nucleosome structure is required, as DNA titration experiments have little effect on the relative level of in vitro mitotic repression. Rather, in vitro repression appears to result largely from phosphorylation of the transcriptional machinery. Depletion experiments indicate that the cdc2 kinase (MPF) is required to initiate transcription repression, but not to maintain it in a cdc2-depleted extract. Subsequent experiments suggest that cdc2 kinase can activate a secondary kinase to carry out transcriptional repression, although we have not ruled out an additional direct role for cdc2 kinase. If this in vitro system correctly mimics the in vivo situation, as do Xenopus extracts in reproducing many nuclear functions, then we conclude that the establishment of transcriptional repression at mitosis in vivo also results from a significant inhibition of the transcriptional machinery itself.

*References*

Adachi, Y., M. Luke, and U. K. Laemmli. 1991. Chromosome asembly in vitro: topoisomerase II is required for condensation. *Cell.* 64:137–148.

Almouzni, G., M. Mechali, and A. P. Wolffe. 1990. Competition between transcription complex assembly and chromatin formation on replicating DNA. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:573–582.

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.

Binetruy, B., T. Smeal, and M. Karin. 1991. Ha-Ras augments c-Jun activity and stimulates phosphorylation of its activation domain. *Nature (Lond.).* 351:122–127.

Boyle, W. J., T. Smeal, L. H. K. Defize, P. Angel, J. R. Woodgett, M. Karin, and T. Hunter. 1991. Activation of protein kinase C decreases phosphorylation of c-Jun at sites that negatively regulate its DNA-binding activity. *Cell.* 64:573–584.

Bradbury, E. M. 1992. Reversible histone modifications and the chromosome cell cycle. *Bioessays.* 14:9–16.

Bradbury, E. M., R. J. Inglis, and H. R. Matthews. 1974. Control of cell division by lysine rich histone F1 phosphorylation. *Nature (Lond.).* 247:257–260.

Brizuela, L., G. Draetta, and D. Beach. 1987. p13$^{suc1}$ act in the fission yeast cell division cycle as a component of the p34$^{cdc2}$ protein kinase. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:3507–3514.

Brown, D. D. 1984. The role of stable complexes that repress and activate eukaryotic genes. *Cell.* 37:359–365.

Carrara, G., G. Di Segni, A. Otsuka, and G. P. Tocchini-Valentini. 1981. Deletion of the 3' half of the yeast tRNA$^{leu-3}$ gene does not abolish promoter function in vitro. *Cell.* 27:371–379.

Chen, G. L., L. Yang, T. C. Rowe, B. D. Halligan, K. M. Tewey, and L. F. Liu, 1984. Nonintercalative antitumor drugs interfere with the breakagereunion reaction of mammalian DNA topoisomerase II. *J. Biol. Chem.* 259:13560–13566.

Cisek, L. J., and J. L. Corden. 1989. Phosphorylation of RNA polymerase by

the murine homologue of the cell-cycle control protein cdc2. *Nature (Lond.).* 339:679–684.

Cohen, P. 1989. The structure and regulation of protein phosphatases. *Annu. Rev. Biochem.* 58:453–508.

Comai, L., N. Tanese, and R. Tijan. 1992. The TATA-binding protein and associated factors are integral components of the RNA polymerase 1 transcription factor, SL1. *Cell.* 68:965–976.

Corden, J. L. 1990. Tails of RNA polymerase II. *Trends Biochem. Soc.* 15:383–387.

Cormack, B. P., and K. Struhl. 1992. The TATA-binding protein is required for transcription by all three nuclear RNA polymerases in yeast cells. *Cell.* 69:685–696.

Dilworth, S. M., S. J. Black, and R. A. Laskey. 1987. Two complexes that contain histones are required for nucleosome assembly in vitro: role of nucleoplasmin and N1 in Xenopus egg extracts. *Cell.* 51:1009–1018.

Draetta, G., F. Luca, J. Westendorf, and 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.

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.

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.

Edgar, B. A., C. P. Kiehle, and G. Schubiger. 1986. Cell cycle control by nucleo-cytoplasmic ratio in early Drosophila development. *Cell.* 44:365–372.

Edgar, B. A., and G. Schubiger. 1986. Parameters controlling transcriptional activation during early Drosophila development. *Cell.* 44:871–877.

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.

Felsenfeld, G. 1992. Chromatin as an essential part of the transcriptional mechanism. *Nature (Lond.).* 355:219–224.

Felsenfeld, G., and J. D. McGhee. 1986. Structure of the 30 nm chromatin fiber. *Cell.* 44:375–377.

Felts, S. J., P. A. Weil, and R. Chalkey. 1990. Transcription factor requirements for in vitro formation of transcriptionally competent 5S rRNA gene chromatin. *Mol. Cell Biol.* 10:2390–2401.

Fink, K., and G. Turnock. 1977. Synthesis of transfer RNA during the synchronous nuclear division cycle in Physarum polycephalum. *Eur. J. Biochem.* 80:93–96.

Fletcher, C., N. Heintz, and R. G. Roeder. 1987. Purification and characterization of OTF-1, a transcription factor regulating cell cycle expression of a human histone H2b gene. *Cell.* 51:773–781.

Forbes, D. J., M. W. Kirschner, and J. W. Newport. 1983. Spontaneous formation of nucleus-like structures around bacteriophage DNA microinjected into Xenopus eggs. *Cell.* 34:13–23.

Fotedar, R., and J. M. Roberts. 1991. Association of p34 with replicating DNA. *In* Cold Spring Harbor Symposia on Quantitative Biology LVI. 325–333.

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.

Gazit, B., H. Cedar, I. Lerer, and R. Voss. 1982. Active genes are sensitive to deoxyribonuclease I during metaphase. *Science (Wash. DC).* 217:648–650.

Geiduschek, E. P., and G. P. Tocchini-Valentini. 1988. Transcription by RNA polymerase III. *Annu. Rev. Biochem.* 57:873–914.

Glotzer, M., A. W. Murray, and M. W. Kirschner. 1991. Cyclin is degraded by the ubiquitin pathway. *Nature (Lond.).* 349:132–138.

Gottesfeld, J. M. 1986. Novobiocin inhibits RNA polymerase III transcription in vitro by a mechanism distinct from topoisomerase II. *Nucleic Acids Res.* 14:2075–2088.

Gottesfeld, J. M., and L. S. Blommer. 1982. Assembly of transcriptionally active 5S RNA gene chromatin in vitro. *Cell.* 28:781–791.

Heck, M. M., W. N. Hittelman, and W. C. Earnshaw. 1989. In vivo phosphorylation of the 170-kDa form of eukaryotic DNA topoisomerase II. *J. Biol. Chem.* 264:15161–15164.

Hirano, T., and T. J. Mitchison. 1991. Cell cycle control of higher-order chromatin assembly around naked DNA in vitro. *J. Cell Biol.* 115:1479–1489.

Johnson T. C., and J. J. Holland. 1965. Ribonucleic acid and protein synthesis in mitotic HeLa cells. *J. Cell Biol.* 27:565–574.

Johnston, L. H., J. H. M. White, A. L. Johnson, G. Lucchini, and P. Plevani. 1987. The yeast DNA polymerase I transcript is regulated in both the mitotic cell cycle and in meiosis and is also induced after DNA damage. *Nuclei Acids Res.* 15:5017–5030.

Kassavetis, G. A., D. L. Riggs, R. Negri, L. H. Nguyen, and E. P. Geiduschek. 1989. Transcription factor IIIB generates extended DNA interactions in RNA polymerase III transcription complexes on tRNA genes. *Mol. Cell Biol.* 9:2551–2566.

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.

Kerem, B.-S., R. Goitein, G. Diamond, M. Marcus, and H. Cedar. 1984. Map-

ping of DNAase I sensitive regions of mitotic chromosomes. *Cell.* 38:493–499.

Kimelman, D., M. Kirshner, and T. Scherson. 1987. The events of the mid-blastula transition in *Xenopus* are regulated by changes in the cell cycle. *Cell.* 48:399–407.

Kornberg, R. D., and Y. Lorch. 1991. Irresistible force meets immovable object: transcription and the nucleosome. *Cell.* 67:833–836.

Labbe, J. C., A. Picard, G. Peaucellier, J. Cavadore, P. Nurse, and M. Doree. 1989. Purification of MPF from starfish: identification as the H1 histone kinase p34$^{cdc2}$ and a possible mechanism for its periodic activation. *Cell.* 57:253–263.

Laskey, R. A., and G. H. Leno. 1990. Assembly of the cell nucleus. *Trends Genetics.* 6:406–410.

Laskey, R. A., A. D. Mills, and N. R. Morris. 1977. Assembly of SV40 chromatin in a cell-free system from Xenopus eggs. *Cell.* 10:237–243.

Laybourn, P. J., and J. T. Kadonaga. 1991. Role of nucleosomal cores and histone H1 in regulation of transcription by RNA polymerase II. *Science (Wash. DC).* 254:238–245.

Lewin, B. 1990. Driving the cell cycle: M phase kinase, its partners and substrates. *Cell.* 61:743–752.

Lewis, C. D., J. S. Lebkowski, A. K. Daly, and U. Laemmli. 1984. Interphase nuclear matrix and metaphase scaffolding structures. *J. Cell Sci.* Suppl. 1:103–122.

Liu, L. F. 1989. DNA topoisomerase poisons as antitumor drugs. *Annu. Rev. Biochem.* 58:351–375.

Lohka, M. J., and Y. Masui. 1983. Formation in vitro of sperm pronuclei and mitotic chromosomes induced by amphibian ooplasmic components. *Science (Wash. DC).* 220:719–721.

Lohka, M. J., and J. L. Maller. 1985. Induction of nuclear envelope breakdown, chromatin condensation, and spindle formation in cell-free extracts. *J. Cell Biol.* 101:518–523.

Mattoccia, E., I. M. Baldi, D. Gandini-Attardi, S. Ciafre, G. P. Tochini-Valentini. 1988. Site selection by the tRNA splicing endonuclease of *Xenopus laevis. Cell.* 55:731–738.

Miake-Lye, R., and M. W. Kirschner. 1985. Induction of early mitotic events in a cell-free system. *Cell.* 41:165–175.

Millstein, L. S., and J. M. Gottesfeld. 1989. Control of gene expression in eukaryotic cells: lessons from class III genes. *Curr. Opin. Cell Biol.* 1:497–502.

Minshull, J., J. Blow, and T. Hunt. 1989. Translation of cyclin mRNA is necessary for extracts of activated *Xenopus* eggs to enter mitosis. *Cell.* 56:947–956.

Moreno, S., and P. Nurse. 1990. Substrates for p34$^{cdc2}$: in vivo veritas? *Cell.* 61:549–551.

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 (Lond.).* 339:280–286.

Newport, J. 1987. Nuclear reconstitution in vitro: stages of assembly around protein-free DNA. *Cell.* 48:205–217.

Newport, J. W., and M. W. Kirschner. 1984. Regulation of the cell cycle during early *Xenopus* development. *Cell.* 37:731–742.

Newport, J., T. Spann, J. Kanki, and D. Forbes. 1985. The role of mitotic factors in regulating the timing of the midblastula transition in *Xenopus*. Cold Spring Harbor Symposia on Quantitative Biology. Vol. L. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 651–656.

Newport, J., and T. Spann. 1987. Disassembly of the nucleus in mitotic extracts: membrane vesicularization, laminin disassembly, and chromosome condensation are independent processes. *Cell.* 48:219–230.

Otsuka, A., A. De Palis, and G. P. Tocchini-Valentini. 1981. Ribonuclease "XlaI" an activity from *Xenopus* laevis oocytes that excises intervening sequences from yeast transfer ribonucleic acid precursors. *Mol. Cell Biol.* 1:269–280.

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.

Pines, J., and T. Hunter. 1990. p34$^{cdc2}$: the S and M kinase? *New Biol.* 2:389–401.

Pfaller, R., C. Smythe, and J. W. Newport. 1991. Assembly/disassembly of the nuclear envelope membrane: cell cycle-dependent binding of nuclear membrane vesicles to chromatin in vitro. *Cell.* 65:209–217.

Prescott, D. M., and M. A. Bender. 1962. Synthesis of RNA and protein during mitosis in mammalian tissue culture cells. *Exp. Cell Res.* 26:260–268.

Razik, M. A., J. Blanco, and J. M. Gottesfeld. 1989. Pathways of nucleoprotein assembly on 5S RNA genes in a *Xenopus* oocyte S-150 extract. *Nucleic Acids Res.* 17:4117–4130.

Roberge, M., J. Th'ng, J. Hamaguchi, and E. M. Bradbury. 1990. The topoisomerase II inhibitor VM-26 induces marked changes in histone H1 kinase, histone H1 and H3 phosphorylation, and chromosome condensation in G2 phase and mitotic BHK cells. *J. Cell Biol.* 111:1753–1762.

Roberts, S. B., N. Segil, and N. Heintz. 1991. Differential phosphorylation of transcription factor Oct1 during the cell cycle. *Science (Wash. DC).* 253:1022–1026.

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.

Schultz, M. C., R. H. Reeder, S. Hahn. 1992. Variants of the TATA-binding protein can distinguish subsets of RNA polymerase I, II, and III promoters. *Cell.* 69:697–702.

Segil, N., S. B. Roberts, and N. Heintz. 1991. Mitotic phosphorylation of the Oct-1 homeodomain and regulation of Oct-1 DNA binding activity. *Science (Wash. DC).* 254:1814–1816.

Shermoen, A. W., and P. H. O'Farrell. 1991. Progression of the cell cycle through mitosis leads to abortion of nascent transcripts. *Cell.* 67:303–310.

Shimamura, A., M. Sapp, A. Rodriguez-Campos, and A. Worcel. 1989. Histone H1 represses transcription from minichromosomes assembled in vitro. *Mol. Cell Biol.* 9:5573–5584.

Simanis, V., and P. Nurse. 1986. The cell cycle control gene cdc2$^+$ of fission yeast encodes a protein kinase potentially regulated by phosphorylation. *Cell.* 45:261–268.

Simpson, R. T., F. Thoma, and J. M. Brubaker. 1985. Chromatin reconstituted from tandemly repeated cloned DNA fragments and core histones: a model system for study of higher order structure. *Cell.* 42:799–808.

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.

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.

Solomon, M. J., M. Glotzer, T. H. Lee, M. Philippe, and M. W. Kirschner. 1990. Cyclin activation of p34$^{cdc2}$. *Cell.* 63:1013–1024.

Tayoda, T, and A. P. Wolffe. 1992. In vitro transcription by RNA polymerase II in extracts of *Xenopus* oocytes, eggs, and somatic cells. *Anal. Biochem.* 203:340–347.

Uemura, T., H. Ohkura, Y. Adachi, K. Morino, K. Shiozaki, and M. Yanagida. 1987. DNA topoisomerase II is required for condensation and separation of mitotic chromosomes in S. pombe. *Cell.* 50:917–925.

van Holde, K. E. 1988. Chromatin structure and transcription. *In* Chromatin. Rich, A., editor. Springer-Verlag, NY. 355–408.

Weintraub, H. 1985. Assembly and propagation of repressed and derepressed chromosomal states. *Cell.* 42:705–711.

White, J. H. M., S. R. Green, D. G. Barker, and L. B. Dumas, and L. H. Johnston. 1987. The CDC 8 transcript is cell cycle regulated in yeast and is expressed coordinately with CDC 9 and CDC 21 at a point preceding histone transcription. *Exp. Cell Res.* 171:223–231.

White, R. J., S. P. Jackson, and P. W. Rigby. 1992. A role for the TATA-box-binding protein component of the transcription factor IID complex as a general RNA polymerase III transcription factor. *Proc. Natl. Acad. Sci.* 89:1949–1953.

Wilson, K. L., and J. Newport. 1988. A trypsin-sensitive receptor on membrane vesicles is required for nuclear envelope formation in vitro. *J. Cell Biol.* 107:57–68.

Wolffe, A. P., and D. D. Brown. 1987. Differential 5S RNA gene expression in vitro. *Cell.* 51:733–740.

Wolffe, A. P., M. T. Andrews, E. Crawford, R. Losa, and D. D. Brown. 1987. Negative supercoiling is not required for 5S RNA transcription in vitro. *Cell.* 49:301–303.

Wolffe, A. P. 1989. Dominant and specific repression of *Xenopus* oocyte 5S genes and satellite I DNA by histone H1. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:527–537.

Wolffe, A. P. 1991. Developmental regulation of chromatin structure and function. Trends Cell Biol. 1:61–66.

Wood, E. R., and W. C. Earnshaw. 1990. Mitotic chromatin condensation in vitro using somatic cell extracts and nuclei with variable levels of endogenous topoisomerase II. *J. Cell Biol.* 111:2839–2850.

Wu, M., and J. G. Gerhart. 1980. Partial purification and characterization of maturation-promoting factor from eggs of *Xenopus* laevis. *Dev. Biol.* 79:465–477.