14. The cleavage of the 21-nucleotide oligomer by the hammerhead ribozyme produces a 12-nucleotide fragment with a 2′,3′–cyclic phosphate and a 9-nucleotide fragment with a free 5′-hydroxyl that can be labeled by T4 polynucleotide kinase and [γ-^{32}P]ATP. The experiment was done as follows: After the 5′-labeled substrate was cleaved by the U6-U4 RNA complex as described, it was desalted, lyophilized, and relabeled with T4 kinase (*11*). The cleavage products were analyzed on a 20%-polyacrylamide denatured gel.
15. J.-P. Perreault, D. Labuda, N. Usman, J.-H. Yang, R. Cedergren, *Biochemistry* **30**, 4020 (1991).
16. S. S. Reid and J. A. Cowan, *ibid.* **29**, 6025 (1990); T. Marciniec *et al.*, *Acta. Biochim. Pol.* **39**, 183 (1989).
17. C. Bark, K. Hammartrom, G. Westin, U. Pettersson, *Mol. Cell. Biol.* **5**, 943 (1985). Also, with regard to the U4 RNA domain, there are three and four A's after position 64 for the best known yeast and human sequences (*3, 9*). Mutation analyses show that insertion of two A's in this region of the hammerhead ribozyme has little effect on the cleavage activity [C. C. Sheldon and R. H. Symons, *Nucleic Acids Res.* **17**, 5679 (1989)].
18. D. E. Ruffner, G. D. Stormo, O. C. Uhlenbeck, *Biochemistry* **29**, 10695 (1990).
19. D. E. Ruffner and O. C. Uhlenbeck, *Nucleic Acids Res.* **18**, 6025 (1990); D. W. Celander and T. C. Cech, *Science* **251**, 401 (1991); D. Smith *et al.*, *J. Biol. Chem.* **267**, 2429 (1992); T. A. Steitz and J. A. Steitz, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 6498 (1993); T. Uchimaru *et al.*, *FASEB J.* **7**, 137 (1993).
20. A. Hampel, R. Tritz, M. Hicks, P. Cruz, *Nucleic Acids Res.* **18**, 299 (1990).
21. F. Michel *et al.*, *Gene* **82**, 5 (1989).
22. H. Sawa and Y. Shirmura, *Genes Dev.* **6**, 244 (1992); H. Sawa and J. Abelson, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11269 (1992).
23. R. Cedergren, F. Lang, D. Gravel, *FEBS Lett.* **226**, 63 (1987).
24. K. A. Jarrell, R. C. Dietrich, P. S. Perlman, *Mol. Cell. Biol.* **8**, 2361 (1988).
25. B. J. Wassarman and R. C. Collins, *Cell* **61**, 685 (1990).
26. M. J. Moore and P. A. Sharp, *Nature* **365**, 364 (1993).
27. M. A. Innis, D. H. Gelfand, J. J. Sninsky, T. J. White, Eds., *PCR Protocols: A Guide to Methods and Applications* (Academic Press, San Diego, CA, 1990), pp. 3–12.
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Mitotic Repression of RNA Polymerase III Transcription in Vitro Mediated by Phosphorylation of a TFIIIB Component

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Interphase cytosol extracts prepared from *Xenopus laevis* eggs are active in RNA polymerase III (Pol III) transcription. Addition of recombinant B1 cyclin to these extracts activates mitotic protein kinases that repress transcription. Affinity-purified p34^{cdc2}–cyclin B kinase (mitosis-promoting factor) is sufficient to effect this repression in a simplified Pol III transcription system. This mitotic repression involves the direct phosphorylation of a component of the Pol III transcription initiation factor TFIIIB, which consists of the TATA box–binding protein (TBP) and associated Pol III–specific factors. The transcriptional activity of the TFIIIB-TBP fraction can be modulated in vitro by phosphorylation with mitotic kinases and by dephosphorylation with immobilized alkaline phosphatase.

Nuclear RNA transcription becomes repressed when eukaryotic cells enter mitosis (*1*). Studies have observed both general mitotic repression of transcription and mitotic repression of specific genes transcribed by RNA polymerase II or III (*2, 3*). Mitotic repression of Pol III transcription can be reproduced in vitro (*4*) with the use of *Xenopus* egg extracts that can easily be shifted from interphase to mitosis (*5–7*). Interphase egg extracts are active in the transcription of Pol III–transcribed genes (*4, 8*), including genes encoding 5S RNA and tRNAs. In contrast, mitotic extracts, generated by the conversion of interphase cytosol to mitosis by means of purified recombinant B1 cyclin (*4, 6, 7, 9, 10*), are repressed in transcription (*4*). Mitotic repression in vitro does not require mitotic chromosome condensation, nucleosome assembly, or the binding of a general repressor protein (*4*). Instead, mitotic repression occurs even in a simplified Pl III transcription system when a mitotic kinase fraction of an egg extract is added (*4*). Action of one or more mitotic kinases is essential for inhibition because the kinase inhibitor 6-dimethylaminopurine (DMAP) blocks inhibition (*4*). Thus, mitotic repression of transcription in vitro involves the direct phosphorylation of the transcriptional machinery.

To elucidate the kinase or kinases that mediate mitotic repression in the *Xenopus* Pol III system, we used partially purified Pol III transcription factors, Pol III (*11*), and *Xenopus* mitotic kinases isolated by p13-agarose affinity chromatography (*4, 12*). The yeast p13*suc1* gene product binds the mitotic *cdc2*–cyclin B kinase (mitosis-promoting factor, MPF) and related kinases (*13*). When a mixture of transcription factors (TFIIIA, TFIIIB, TFIIIC, and Pol III) was incubated with mitotic kinases bound to p13-agarose beads and the beads were subsequently removed by centrifugation, the transcription of 5S DNA was repressed (Fig. 1A, lane 1). Similar repression of *Xenopus* tRNA^{Met1} and tRNA^{Tyr} transcription was observed (*14*). This inhibition could be prevented by including the kinase inhibitor DMAP in the reaction (lane 4) or by substituting adenosine triphosphate (ATP) with the nonhydrolyzable analog adenylyl-imidodiphosphate (AMP-PNP) (lane 3). In contrast, p13-agarose bound with interphase egg extract proteins had no effect on transcription (lane 2). Thus, immobilized *cdc2*-cyclin kinase and related mitotic kinases directly repress transcription.

Purified *cdc2*–cyclin B kinase alone caused mitotic repression in the reconstituted transcription system. The *cdc2* kinase was purified from a cyclin-activated mitotic extract by glutathione-Sepharose chromatography with the glutathione-S-transferase tag present on the recombinant cyclin B1 protein (*4, 6, 9, 10, 12*); the immobilized *cdc2*–cyclin B kinase inhibited 5S gene transcription (Fig. 2B, lane M).

To identify the target of the mitotic kinase, we performed a rescue experiment. A mixture of the transcription factors was treated with either interphase or mitotic p13-agarose in the presence of ATP, and the beads were removed after incubation. Factors treated with mitotic beads did not support transcription of the 5S RNA gene (Fig. 1B, lane 5). Each of the chromatographic fractions needed for Pol III transcription (and not exposed to the p13-bound kinase) was added back to separate reactions. DMAP was included to ensure that any secondary kinase activity present in the original factor mixture would not phosphorylate the added untreated factor or factors. Addition of the phosphocellulose fraction PC-B, which contains TFIIIB and Pol III, fully restored transcription of the 5S RNA gene (Fig. 1B, lane 7). Neither TFIIIA (lane 6) nor TFIIIC (lane 8) gave significant rescue of transcription (*15*). The same effect was observed with the tRNA^{TyrD} gene template (*14*). These results suggest that the target of the mitotic kinase is a component of the PC-B fraction and that the relevant factor or factors support transcription in the nonphosphorylated forms and are inactive in the phosphorylated forms.

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To determine whether the TFIIIB present in the PC-B fraction is the target of mitotic repression, we further purified TFIIIB. The PC-B fraction was chromatographed on DEAE-Sephadex and Mono Q fast protein liquid chromatography (FPLC) (*16*). The results of a 5S gene transcription experiment in which we used TFIIIA, TFIIIC, and the phosphocellulose fraction PC-C (as a source of Pol III) to assay TFIIIB activity after further purification are shown (Fig. 2A). No specific transcription was observed in the absence of TFIIIB (lane A + C), whereas the fraction eluted from DEAE-Sephadex with 0.15 M ammonium sulfate provided an active source of TFIIIB (lane denoted Input). Upon further fractionation, TFIIIB activity eluted from Mono Q at ~0.31 M KCl (Fig. 2A, fractions 19 through 21). When the complete reaction (*16*) was treated with immobilized *cdc2* kinase, transcription was eliminated (Fig. 2B, lane M). We rescued the 5S transcription by adding either the DEAE-Sephadex TFIIIB (Fig. 2B, input) or the Mono Q TFIIIB fractions to the mitotically repressed reaction (Fig. 2B, fractions 19 through 21). Both TFIIIB (Fig. 2A) and the rescue activities (Fig. 2B) co-eluted from the Mono Q resin, which indicates that the target of the mitotic kinase involved in repression of Pol III transcription is an integral component or components of TFIIIB.

The TATA box–binding protein (TBP), which plays a central role in Pol II transcription, is also required for Pol I and Pol III transcription (*17, 18*). For Pol III transcription, TBP and the associated Pol III–specific factors (TAFs) form TFIIIB (*19*). We assayed the Mono Q fractions for the TBP component of TFIIIB with a gel mobility shift assay using a radiolabeled double-stranded TATA box oligonucleotide. As expected, TATA box DNA binding activity in the Mono Q fractions co-purified with TFIIIB transcriptional activity (Fig. 2C, lanes 19 and 20). We also found that Pol III transcription in *Xenopus* oocyte S-150 extracts was abolished by TBP-TAF depletion, which we accomplished by using a TATA box DNA–Sepharose resin (*20*); transcription was restored to the depleted fraction by the addition of either Mono Q–purified TFIIIB or the protein fraction eluted from the TATA-Sepharose resin. A similar protein fraction from a control B-block DNA-Sepharose resin or TFIIIC did not restore activity to the TATA-Sepharose–depleted extract (*20*). This indicates that TFIIIB is retained on the TATA-Sepharose resin.

To determine whether the target of mitotic repression resides in the TATA-binding fraction, a complete transcription mix was first incubated with mitotic p13-agarose. When this repressed reaction was supplemented with increasing amounts of TATA box DNA-Sepharose–binding proteins isolated from interphase egg cytosol, the TATA-binding fraction rescued 5S gene transcription (Fig. 3A, lanes 6 to 8). We also tested the TATA-Sepharose–binding proteins from both interphase and mitotic extracts using the $tRNA^{TyrD}$ gene as the template (Fig. 3B). Only the TATA-binding fraction from the interphase extract rescued transcription (Fig. 3B, lanes 9 and 10). The same fraction from the mitotic extract did not restore transcription (lanes 7 and 8). The mitotic TATA-binding fraction did not inhibit transcription in interphase p13-treated samples (lanes 3 and 4). These data suggest that TBP or a TBP-associated component of TFIIIB from the interphase extract rescues transcription and that the equivalent protein or proteins in the mitotic extract are phosphorylated and unable to rescue transcription.

To test this hypothesis, we treated the interphase TBP-TAFs with either interphase or mitotic p13-agarose and then asked whether these treated fractions could still restore transcription to the mitotically inhibited transcription reaction. The TATA-binding fraction treated with interphase p13-agarose res-

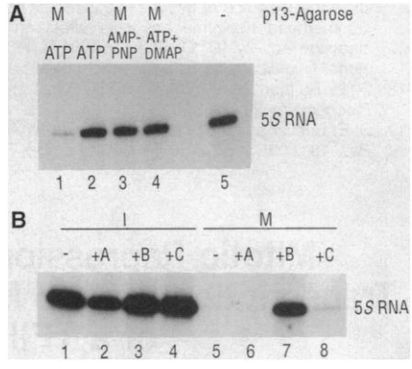

Fig. 1. (**A**) Repression of transcription can be mediated by a mitotic kinase bound to p13-agarose. A mixture of TFIIIA, PC-B TFIIIB, TFIIIC, and Pol III (*11*) was treated with mitotic kinases (M) bound to p13-agarose beads (lanes 1, 3, and 4) or with p13-agarose bound with interphase (I) extract proteins (lane 2) (*12*). Reactions were in the presence of 1 mM ATP (lanes 1 and 2), 1 mM AMP-PNP (lane 3), or 1 mM ATP plus 2.5 mM DMAP (lane 4). After incubation on a rotator for 1 hour at 4°C, the agarose beads were pelleted, and 100 ng of 5S plasmid DNA (*27*), along with labeled and unlabeled nucleoside triphosphates, was added to the supernatants. Transcription products were analyzed after a subsequent 2-hour incubation. Lane 5 shows the products of a control (untreated) reaction. (**B**) The phosphocellulose TFIIIB–Pol III fraction can rescue mitotic inhibition of 5S transcription. Mixtures of TFIIIA, PC-B TFIIIB, and TFIIIC were treated with interphase proteins bound to p13-agarose (lanes 1 through 4) or with mitotic kinases bound to p13-agarose (lanes 5 through 8). After pelleting the beads, we made the following additions to the supernatants: lanes 1 and 5, 2 μl of buffer; lanes 2 and 6, 18 ng of TFIIIA (+A); lanes 3 and 7, 2 μl of TFIIIB–Pol III fraction (+B; 6 μg of protein); and lanes 4 and 8, 1 μl (50 fmol) of TFIIIC (+C). All reactions contained 5S plasmid DNA, 2.5 mM DMAP, and nucleotides in the subsequent 2-hour incubation.

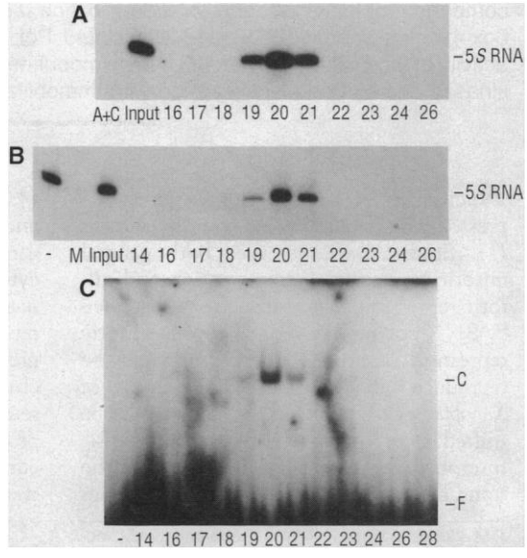

Fig. 2. Mono Q FPLC purification of TFIIIB and rescue of mitotic repression of transcription. Phosphocellulose TFIIIB was fractionated on DEAE-Sephadex and Mono Q FPLC as described (*16*). (**A**) TFIIIB transcriptional activity was monitored in a system containing 5S DNA, TFIIIA, TFIIIC, and Pol III (lane denoted A + C). These reactions (*16*) were supplemented with 2.5 μl of the DEAE-Sephadex TFIIIB fraction (Mono Q input) or with 2.5-μl aliquots of the indicated Mono Q fractions eluting between 0.25 and 0.4 M KCl. (**B**) Rescue of mitotic repression with Mono Q fractions. A mixture of TFIIIA, DEAE-Sephadex TFIIIB, TFIIIC, and Pol III (*16*) was treated with $p34^{cdc2}$–cyclin B–GST fusion protein activated in the egg extract and bound to glutathione-Sepharose (*13*). After pelleting the Sepharose beads, we made the following additions: 2.5 μl of buffer (lane M), 2.5 μl of DEAE-Sephadex TFIIIB (Mono Q Input), or 2.5 μl of the indicated Mono Q fractions. The control lane (−) has a reaction that was not treated with the kinase. (**C**) TATA box DNA gel mobility shift with a 26–base pair double-stranded TATA box oligonucleotide that was radiolabeled and 2.5-μl aliquots of the indicated Mono Q fractions in a 20-μl binding reaction. We used a 6% nondenaturing gel run in 88 mM tris-borate buffer (pH 8.3) to resolve complexes (C) from free oligonucleotide (F).

cued transcription (Fig. 4A, lane 3); however, the interphase TATA-binding fraction subjected to mitotic kinase phosphorylation could not restore transcription (Fig. 4A, lane 4). Rescue activity was observed if mitotic phosphorylation was performed in the presence of DMAP (lane 5), which affirms that it is the enzymatic action of a mitotic protein kinase that inactivates a required factor. These results reinforce the conclusion that the target of the mitotic kinase resides in TBP or in a TBP-associated component of TFIIIB, which is active in the interphase but which becomes phosphorylated to an inactive state during mitosis. The data predict that in the normal cell cycle this factor should be reactivated by dephosphorylation after exit from mitosis. To test this hypothesis, we treated the mitotic TATA-Sepharose–binding fraction with alkaline phosphatase–agarose beads (*21*). Phosphatase treatment restored transcriptional rescue activity to the mitotic TATA-Sepharose–binding fraction (Fig. 4B, lane 6). Similar treatment of the interphase TATA-binding fraction slightly enhanced its ability to restore transcription (lane 4). Our results are consistent with inactivation of TBP or a TBP-associated protein by phosphorylation (*22*).

Previous work with total *Xenopus* egg cytosol found that, after depletion of *cdc2*–cyclin B kinase with p13-Sepharose, a secondary mitotic protein kinase or kinases could cause repression of Pol III transcription (*4*). The secondary kinase or kinases were activated by mitotic conversion of the extract with B1 cyclin. We show here that affinity-purified *cdc2*–cyclin B kinase can repress transcription in a simplified Pol III system. These results indicate that MPF can directly phosphorylate the transcriptional machinery. We suggest that in vivo both MPF and a secondary protein kinase activated by MPF are involved in mitotic repression of Pol III transcription.

Our results further indicate that the TFIIIB TBP-TAF complex is a major target of the mitotic kinase or kinases. Mitotic phosphorylation of this protein complex down-regulates Pol III transcription. This down-regulation can be reversed by dephosphorylation with alkaline phosphatase. Because TFIIIB is required for initiation of Pol III transcription (*23*), inactivation of this factor would result in reduced initiation events during the mitotic phase of the cell cycle. Phosphorylation of TBP or the TAFs or both during mitosis could effect a global repression of all polymerases observed during mitosis because each polymerase uses TBP and TAFs in the formation of transcription complexes (*17, 18*). Studies of Pol II transcription describe other proteins that might show cell cycle regulation and could act through the TBP component of Pol II transcription factor TFIID (*24*). Pol II transcription in *Xenopus* egg extracts has been observed (*25*), and a strategy similar to the one used here may work to determine whether Pol II transcription is mitotically inactivated in vitro and, if so, the Pol II–specific target of repression.

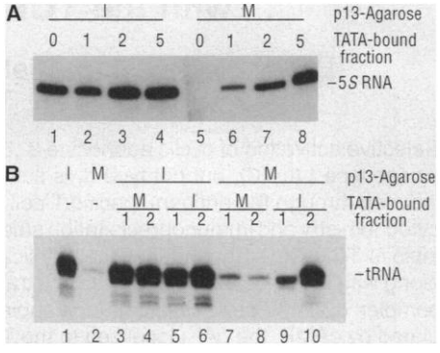

Fig. 3. The TATA-binding protein or an associated factor is the target of the mitotic kinase. Transcription reactions were performed with the 5*S* gene (**A**) or with the $tRNA^{TyrD}$ gene (**B**) (*27*). Mixtures of transcription factors and polymerase (*11*) were first treated with mitotic (M) or interphase (I) p13-agarose (*12*). After the p13 beads were pelleted, the reactions were supplemented in (A) with buffer (lanes 1 and 5) or with the indicated volumes (in microliters) of TATA-Sepharose–binding proteins isolated from the interphase extract (lanes 2 through 4 and 6 through 8) (*20*). At that time, the 5*S* gene or tRNA gene template was added and transcription was assayed (*11*). In (B), the experiment was performed in a similar fashion except that 1- and 2-μl aliquots of the TATA-Sepharose–binding fraction isolated from either the interphase or mitotic extracts were added as indicated. For all reactions, transcripts were analyzed after a subsequent 2-hour incubation in the presence of 2.5 mM DMAP and nucleoside triphosphates.

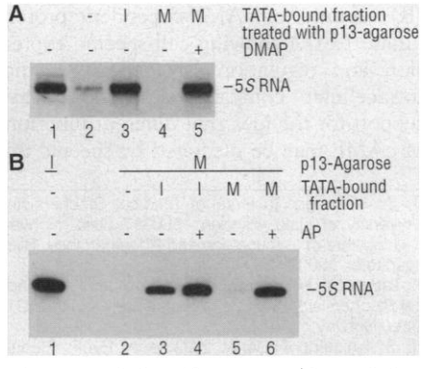

Fig. 4. Inactivation of the TATA-Sepharose–binding proteins with a mitotic kinase and reactivation with alkaline phosphatase. (**A**) Transcription reaction mixtures (*11*) were treated with mitotic p13-agarose (lanes 2 through 5). Lane 1 shows a control (untreated) reaction. In lanes 3 through 5, reactions were supplemented with interphase TATA-Sepharose–binding proteins (2 μl) that were treated with interphase (I) or mitotic (M) p13-agarose (in the presence of ATP). For the reaction shown in lane 5, this treatment was in the presence of 2.5 mM DMAP. (**B**) Activation of the mitotic TATA-Sepharose–binding fraction by treatment with immobilized alkaline phosphatase (AP) (*21*). The mixture of transcription factors was treated with interphase or mitotic p13-agarose. After pelleting the p13 beads, we supplemented the reactions with 5 μl of TATA-Sepharose–binding proteins isolated from either the mitotic or interphase extracts. Before addition, aliquots of these factors were treated with calf intestinal AP coupled to agarose beads. For both panels, the 5*S* gene template was then added, and transcripts were analyzed after a subsequent 2-hour incubation in the presence of nucleoside triphosphates.

REFERENCES AND NOTES

1. D. M. Prescott and M. A. Bender, *Exp. Cell Res.* **26**, 260 (1962); J. Taylor, *Ann. N.Y. Acad. Sci.* **90**, 409 (1960).
2. K. Fink and G. Turnock, *Eur. J. Biochem.* **80**, 93 (1977); T. C. Johnson and J. J. Holland, *J. Cell Biol.* **27**, 565 (1965); L. H. Johnston, J. H. M. White, A. L. Johnson, G. Lucchini, P. Plevani, *Nucleic Acids Res.* **15**, 5017 (1987); B. A. Edgar and G. Schubiger, *Cell* **44**, 871 (1986); D. M. Kimmelman, M. Kirschner, T. Scherson, *ibid.* **48**, 399 (1987).
3. A. W. Shermoen and P. H. O'Farrell, *Cell* **67**, 303 (1991).
4. P. Hartl, J. Gottesfeld, D. J. Forbes, *J. Cell Biol.* **120**, 613 (1993).
5. R. Miake-Lye and M. W. Kirschner, *Cell* **41**, 165 (1985).
6. A. W. Murray, M. J. Solomon, M. W. Kirschner, *Nature* **339**, 280 (1989).
7. V. J. Allan and R. D. Vale, *J. Cell Biol.* **113**, 347 (1991); T. Hirano and T. J. Mitchison, *ibid.* **115**, 1479 (1991).
8. G. Almouzni, M. Mechali, A. P. Wolffe, *EMBO J.* **9**, 573 (1990); *Mol. Cell. Biol.* **11**, 655 (1991).
9. M. J. Solomon, M. Glotzer, T. H. Lee, M. Philippe, M. W. Kirschner, *Cell* **63**, 1013 (1990).
10. C. Smythe and J. Newport, *ibid.* **68**, 787 (1992).
11. Partial purification of transcription factors TFIIIB, Pol III, and TFIIIC from *Xenopus* oocyte S-150 extracts [G. C. Gliken, I. Ruberti, A. Worcel, *Cell* **37**, 33 (1984)] was carried out by phosphocellulose chromatography as described [J. Segall, T. Matsui, R. Roeder, *J. Biol. Chem.* **255**, 11986 (1980)]. TFIIIC was further purified by B-block DNA-Sepharose affinity chromatography [H. J. Keller, Q. You, P. J. Romaniuk, J. M. Gottesfeld, *Mol. Cell. Biol.* **10**, 5166 (1990)] and contained 50 fmol of B-block oligonucleotide binding activity per microliter. TFIIIA was purified from immature oocytes [D. R. Smith, I. J. Jackson, D. D. Brown, *Cell* **37**, 645 (1984)]. Transcription reactions (20 μl) contained 6 μl of the fraction eluting from the phosphocellulose P11 column at 0.35 M KCl (containing TFIIIB and Pol III; ~16 μg of total protein), 1 to 2 μl of TFIIIC, and for the 5*S* RNA gene reactions, 9 ng of TFIIIA (yielding a fivefold excess of TFIIIA over 5*S* genes). For 5*S* RNA gene reactions, TFIIIA and DNA were incubated together for 30 min before the addition of the other reaction components. Nucleotides were included at final concentrations of 0.6 mM for ATP, uridine triphosphate, and cytidine triphosphate; guanosine triphosphate (GTP) was included at a concentration of 0.02 mM along with 10 μCi of [α-^{32}P]GTP. Reactions were performed for 2 hours with a final concentration of 12 mM Hepes-

OH (pH 7.5), 60 mM KCl, 6 mM $MgCl_2$, 25 μM $ZnCl_2$, and glycerol at 6 to 8% (v/v) (transcription buffer).
12. The p13-agarose beads (Oncogene Science, Santa Cruz Biotechnology, Santa Cruz, CA) were washed three times in transcription buffer (*11*) before incubation with an equal volume of either the mitotic or interphase egg cytosol extracts (*4*) on a rotator for 1 to 2 hours at 4°C. The activated *cdc2*–cyclin B kinase–glutathione-S-transferase (GST) from the mitotic extract was affinity-purified on glutathione-Sepharose (Pharmacia) as described by Solomon and co-workers (*9*). Binding of protein kinase activity from the mitotic extract to p13-agarose or glutathione-Sepharose was confirmed by phosphorylation of histone H1 with [γ-^{32}P]ATP [C. Smythe and J. W. Newport, *Methods Cell Biol.* **35**, 449 (1991)]. For phosphorylation of transcription factors, one-half volume of packed beads was used per volume of transcription factor fractions in the presence of unlabeled nucleoside triphosphates (as for a transcription reaction). Samples were incubated on a rotator at ambient temperature for 1 hour, and the beads were pelleted in a microfuge. The supernatants were then transferred to fresh tubes for the subsequent assay of the transcription activity of the treated factors.
13. W. G. Dunphy, L. Brizuela, D. Beach, J. Newport, *Cell* **54**, 423 (1988); L. Brizuela, G. Draetta, D. Beach, *EMBO J.* **6**, 3507 (1987); F. Fang and J. Newport, *Cell* **66**, 731 (1991).
14. V. J. Wolf and J. M. Gottesfeld, unpublished data.
15. Addition of TFIIIC resulted in a small amount of rescue, but this observation could be the result of the limitation of this factor in the reconstituted system.
16. The phosphocellulose TFIIIB fraction was dialyzed and sequentially fractionated on DEAE-Sephadex and Mono Q FPLC as described (*26*) with the exception that 0.15 M ammonium sulfate was used for elution of TFIIIB from the DEAE resin. DEAE-Sephadex TFIIIB (1 ml; 220 μg) was applied to a 1-ml Mono Q column, and 40 1-ml fractions were collected between 0.1 and 0.6 M KCl in buffer D (*26*). TFIIIB activity was assayed in 25-μl reactions containing TFIIIA, TFIIIC, 4 μl of the PC-C fraction (12 μg of protein) as a source of Pol III, and other components as in (*11*). The PC-C fraction was used as a source of polymerase because Pol III is separated from TFIIIB by DEAE-Sephadex chromatography. For rescue assays, the complete transcription mixture contained the components listed above and 2.5 μl of DEAE-Sephadex TFIIIB (0.7 μg of protein).
17. R. J. White, S. P. Jackson, P. W. Rigby, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1949 (1992).
18. L. Comai, N. Tanese, R. Tjian, *Cell* **68**, 965 (1992); B. P. Cormack and K. Struhl, *ibid.* **69**, 685 (1992); M. C. Schultz, R. H. Reeder, S. Hahn, *ibid.*, p. 697.
19. S. M. Lobo, M. Tanaka, M. L. Sullivan, N. Hernandez, *ibid.* **71**, 1029 (1992); A. K. P. Taggart, T. S. Fisher, B. F. Pugh, *ibid.*, p. 1015; R. J. White and S. P. Jackson, *ibid.*, p. 1041; G. A. Kassavetis *et al.*, *ibid.*, p. 1055.
20. Ligated multimers of a 26–base pair double-stranded oligonucleotide corresponding in sequence to the adenovirus major late promoter TATA box (*17*) or a control 24–base pair oligonucleotide containing the consensus Pol III B-block sequence [H. J. Keller, P. J. Romaniuk, J. M. Gottesfeld, *J. Biol. Chem.* **267**, 18190 (1992)] were coupled to cyanogen bromide–activated Sepharose 6B-Cl as described by the supplier (Sigma). Before use, the resin was washed several times with transcription buffer containing 1 mM dithiothreitol and 1 mM $MgCl_2$. Equal volumes of either the mitotic or interphase extracts and packed DNA-Sepharose were mixed in Eppendorf tubes on a rotator for 1 hour, and unbound proteins were removed by several washes with the same buffer. Bound proteins were eluted with this buffer containing 0.5 M KCl, and we reduced the KCl concentration to 0.1 M by dialysis for 6 to 14 hours at 4°C using a Pierce (Rockford, IL) microdialyzer. The final concentration of TATA-binding proteins from either extract was approximately 100 ng/μl; this concentration represents <0.5% of the starting cytosol protein. The TATA-Sepharose–binding fraction from interphase extracts contained TFIIIB activity (as detected in a complementation assay with purified TFIIIA and the PC-C fraction), but had no transcriptional activity either alone or in combination with purified TFIIIA and TFIIIC, indicating that Pol III is absent from the TATA-binding fraction.
21. Agarose beads coupled with alkaline phosphatase (6.2 units per milligram of beads; Sigma) were washed three times with transcription buffer (*11*), and equal volumes of packed beads and transcription factors or extracts were incubated on a rotator for 1 hour at ambient temperature. The beads were pelleted in a microfuge, and the supernatants were then tested for transcriptional activity.
22. Protein phosphorylation experiments suggest that TBP itself is not a substrate for direct phosphorylation by the *cdc2*–cyclin B kinase (*14*); no protein of the expected molecular mass of *Xenopus* TBP [33 kD; S. Hashimoto *et al.*, *Nucleic Acids Res.* **20**, 3788 (1992)] is phosphorylated in the TFIIIB-TATA–binding fractions. Furthermore, the deduced amino acid sequence of *Xenopus* TBP does not contain the consensus sequence for phosphorylation by *cdc2* kinase, and recombinant *Xenopus* TBP is not a substrate for phosphorylation by the purified kinase (A. Leresche and J. M. Gottesfeld, unpublished data). In contrast, several other polypeptides in the TFIIIB-TAF fraction are phosphorylated in vitro by *cdc2* kinase.
23. G. A. Kassavetis, B. R. Braun, L. H. Nguyen, E. P. Geiduschek, *Cell* **60**, 235 (1990).
24. S. Rupert, E. H. Wang, R. Tjian, *Nature* **362**, 175 (1993); K. Hisatake *et al.*, *ibid.*, p. 179; J. A. Inostroza, F. H. Mermelstein, W. S. Lane, D. Reinberg, *Cell* **70**, 477 (1992).
25. T. Tayoda and A. P. Wolffe, *Anal. Biochem.* **203**, 340 (1992).
26. K. A. Simmen, J. Bernues, J. D. Lewis, I. W. Mattaj, *Nucleic Acids Res.* **20**, 5889 (1992).
27. Plasmids containing Pol III–transcribed sequences from *Xenopus laevis* have been described: the somatic-type 5*S* RNA gene [pXIs 11; R. C. Peterson, J. L. Doering, D. D. Brown, *Cell* **20**, 131 (1980)] and the tRNA gene for TyrD [F. Stutz, E. Gouillaud, S. G. Clarkson, *Genes Dev.* **3**, 1190 (1989)].
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Location of cAMP-Dependent Protein Kinase Type I with the TCR-CD3 Complex

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Selective activation of cyclic adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase type I (cAKI), but not type II, is sufficient to mediate inhibition of T cell replication induced through the antigen-specific T cell receptor–CD3 (TCR-CD3) complex. Immunocytochemistry and immunoprecipitation studies of the molecular mechanism by which cAKI inhibits TCR-CD3–dependent T cell replication demonstrated that regulatory subunit Iα, along with its associated kinase activity, translocated to and interacted with the TCR-CD3 complex during T cell activation and capping. Regulatory subunit IIα did not. When stimulated by cAMP, the cAKI localized to the TCR-CD3 complex may release kinase activity that, through phosphorylation, might uncouple the TCR-CD3 complex from intracellular signaling systems.

The demonstration of multiple regulatory (R) subunits of cAMP-dependent protein kinase (cAK) showing cell-specific expression and regulation, as well as distinct intracellular compartmentalization, gave support for the idea that different functions of cAMP may be mediated by specific isozymes of cAK (*1*). In human peripheral blood T lymphocytes, the cAKI holoenzyme [composed of two RIα subunits and two catalytic (C) subunits ($RI\alpha_2C_2$)] is soluble, whereas cAK type II (cAKII) ($RII\alpha_2C_2$) is particulate (*2*). Furthermore, activation of cAKI, but not cAKII, is sufficient to mediate the inhibitory effect of cAMP on TCR-CD3–induced T cell replication (*2*). To investigate the possible mechanism for cAKI-mediated inhibition of TCR-CD3–induced T cell replication, we examined the location of cAKI and cAKII in quiescent cells and in cells stimulated through the TCR-CD3 complex.

The subcellular localization of cAKI and cAKII was assessed with antibodies to RIα (anti-RIα) or RIIα (anti-RIIα) and visualized by indirect immunofluorescence with fluorochrome-labeled secondary antibodies

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