Association of cdk2 Kinase with the Transcription Factor E2F During S Phase

MICHELE PAGANO, GIULIO DRAETTA, PIDDER JANSEN-DÜRR

The transcription factor E2F controls the expression of several proliferation-related genes and is a target of the adenovirus E1A oncogene. In human cells, both cyclin A and the cdk2 protein kinase were found in complexes with E2F. Although the total amounts of cdk2 were constant in the cell cycle, binding to E2F was detected only when cells entered S phase, a time when the cdk2 kinase is activated. These data suggest that the interaction between cdk2 and E2F requires an active kinase that has cyclin A as a targeting component.

COMPONENTS OF THE CELL CYCLE control machinery are likely to be targets of oncogenic events (*1, 2*). Of the many cyclin-dependent kinase (cdk) subunits (*3*) identified in mammalian cells, cyclin A interacts with oncogene products. In virally infected human cells, cyclin A associates with the adenovirus E1A gene product (E1A) (*4, 5*), and the same E1A sequences that were found necessary for E1A to immortalize primary cells are required for cyclin A binding to E1A, suggesting that this interaction is of functional relevance for the oncogenic events mediated by E1A (*6*). The human cyclin A gene was first cloned as the integration site of hepatitis B virus, in a hepatocellular carcinoma (*7*). Cyclin A is a nuclear protein (*8, 9*) that binds two distinct catalytic subunits, cdc2 and cdk2 (*9, 10*). Furthermore, microinjection of antibodies to cyclin A into cells in G1 inhibits those cells from replicating DNA, and microinjection of these antibodies into cells in G2 inhibits entry into mitosis (*9*), suggesting that the two cyclin A complexes are each required for progression through a specific point in the cell cycle. The cyclin A–cdk2 complex could function in controlling initiation of or progression through DNA replication, or both. Therefore, the binding of cyclin A–cdk2 by the E1A protein (*10, 11*) might reflect a requirement for the virus to interfere with the host DNA synthesis apparatus in order to replicate its own genome.

E2F is a cellular factor recruited by the adenovirus for transcription of the viral E2 mRNA (*12*), and E2F-dependent transcription is stimulated by E1A (*13–15*). E2F binding sites are present in the promoter region of several cellular genes (*16–21*). At least two genes carrying E2F binding sites, dihydrofolate reductase (*16*) and human DNA polymerase α (*21*), are essential for DNA replication. Furthermore, in nontransformed mammalian cells, E2F forms a complex with both cyclin A and the retinoblastoma gene product (pRb), and these complexes can be displaced by E1A (*22–25*). The regions of E1A required to activate E2F (*26*) are the same regions that are required for the transforming activity of E1A (*27*), and for binding cellular proteins, such as cyclin A (*6*) or pRb (*27*).

Because cyclin A can bind either cdc2 or cdk2, we investigated whether these proteins were also components of complexes containing E2F. We generated a polyclonal antiserum by injecting rabbits with a peptide corresponding to the COOH-terminus of human cdk2. The affinity-purified antibodies (anti-cdk2) recognized a single band on immunoblots from human cell extracts and did not cross-react with cdc2 (Fig. 1A) (*9*). We investigated whether these antibodies and antibodies to the COOH-terminus of cdc2 (anti-cdc2) (*28*) interfered with the E2F gel retardation assay. HeLa cell extracts were incubated with an oligonucleotide encompassing the sequence of the distal E2F binding site in the adenovirus E2 promoter and then subjected to gel electrophoresis.

M. Pagano and G. Draetta, Differentiation Programme, European Molecular Biology Laboratory, D-6900 Heidelberg, Germany.
P. Jansen-Dürr, Deutsches Krebsforschungzentrum, D-6900 Heidelberg, Germany.

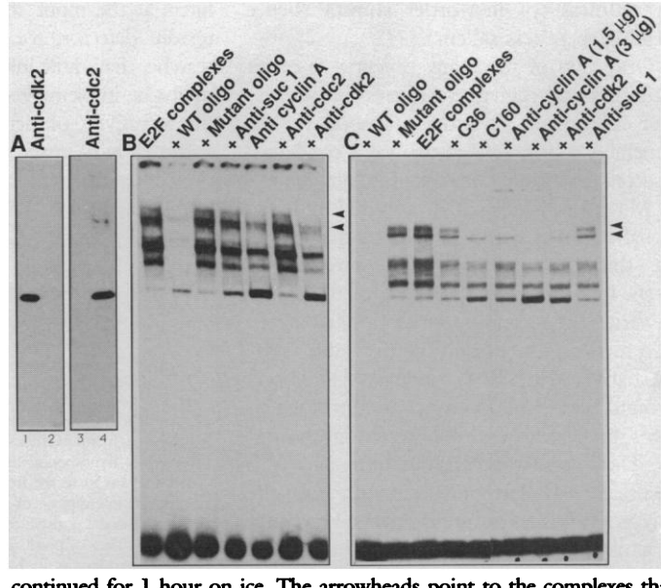


Fig. 1. Detection of human cdk2 and cyclin A in E2F complexes. Specificity of anti-cdk2 (**A**). Proteins (5 μg) from *Escherichia coli* expressing recombinant cdk2 (lanes 1 and 3) or recombinant cdc2 (lanes 2 and 4) were separated by gel electrophoresis and immunoblotted with affinity-purified anti-cdk2 (lanes 1 and 2) or anti-cdc2 (lanes 3 and 4). Band shift assays of HeLa cells (**B**) and WERI cells (pRb-deficient) (**C**). Cells were cultured as described (*4, 34*). Whole cell extracts were prepared by a rapid extraction method (*35*) with some modifications (*15*). Briefly, cells were collected, rinsed with phosphate-buffered saline, and resuspended in 1.5 volumes of lysis buffer A [20 mM Hepes (pH 7.9), 0.4 M NaCl, glycerol 25%, 1 mM EDTA, 2.5 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride]. Cells were kept on ice for 20 min, frozen at −70°C, and thawed on ice. Subsequently the suspension was vigorously mixed and centrifuged for 10 min at 13,600*g* in a microcentrifuge. Band shift assays were performed as follows: synthetic oligonucleotides corresponding to both strands of the distal E2F binding site of the adenovirus E2 promoter (GATCCACTAGTTTCGCGCGCTTTCTA and GATCTAGAAAGCGCGCGAAACTAGTG) were used as wild-type (WT) probe. The mutant oligonucleotides correspond to the sequences GATCCACTAGTTTACTCAGATAACTA and GATCTAGTTATCTGAGTAAACTAGTG, respectively (*36*). The 5′ ends of the oligonucleotides were labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (adenosine triphosphate). The DNA was purified by gel electrophoresis and diluted to 10,000 cpm/μl. Probe DNA (1 μl) was incubated in a final volume of 10 μl with 500 ng of poly-dAdT, 2 mM $MgCl_2$, glycerol (10%), and 2 μl (5 μg) of extract for 10 min at 20°C. The mixture was separated on a native polyacrylamide gel (4.5%) run in 0.3× tris-borate-EDTA (TBE) buffer for 60 to 75 min at 4°C. The gel was dried and exposed for autoradiography. The specificity of the resulting protein complexes was investigated by competition with a 20-fold excess of unlabeled probe DNA of either the wild-type or the mutated sequence, as indicated. To analyze the protein composition of the complexes the reactions were incubated with affinity-purified antibodies (1.5 μg) or monoclonal antibody (0.1 μg). After the 10-min incubation at 20°C the antibody was added and the incubation continued for 1 hour on ice. The arrowheads point to the complexes that were disrupted by the presence of both anti-cdk2 and anti–cyclin A. The preparation, purification, and characterization of the antibodies to COOH-terminus of cdk2 (anti-cdk2), the COOH-terminus of cdc2 (anti-cdc2), cyclin A (anti–cyclin A), and sucl (anti-sucl) have been described (*9, 28, 29*). The monoclonal antibody to cyclin A (C160) has been described (*4*). The C36 monoclonal antibody to pRb (*37*) was obtained from Triton Biosciences Inc.

We detected five specific complexes that were not competed by a mutated E2F oligonucleotide; however the complexes were almost completely absent if excess wild-type oligonucleotide was present in the incubation mixture (Fig. 1B). Addition of affinity-purified anti-cdk2 or anti–cyclin A inhibited the appearance of two specific bands and increased the intensity of a faster migrating band, which might be free E2F (*14*). This band appeared in part to be due to non-specific binding because it was not displaced by excess wild-type oligonucleotide. Affinity-purified anti-cdc2 or antibodies to suc1, another cdc2-associated protein (*29*), did not affect the gel retardation profiles. Incubation with antibodies to cyclin B had no effect on complex formation (*30*). An analogous experiment was performed with extracts from a pRb-deficient cell line (Weri) (Fig. 1C). Addition of affinity-purified anti-cdk2 or anti–cyclin A as well as the monoclonal antibody to cyclin A C160 (*4*) inhibited the appearance of two specific E2F-complexes. Antibodies to pRb, suc1 (Fig. 1C), or to cdc2 or cyclin B (*30*) did not affect the band shift pattern. This experiment demonstrates that both cyclin A and cdk2 can bind E2F-complexes independently of pRb.

To analyze further the presence of cdk2 in E2F complexes, extracts from asynchronously growing HeLa cells were incubated with either affinity-purified anti-cdk2 or anti-cdc2. Immunocomplexes were bound to protein A–Sepharose, washed, and eluted with excess antigenic peptide; the eluted protein complexes were used for band-shift assays. E2F complexes were released only from anti-cdk2 but not anti-cdc2 immunoprecipitates (Fig. 2A). These complexes were not detected after incubation in the presence of excess wild-type oligonucleotide, but were not affected by incubation with mutant oligonucleotide (Fig. 2B). Furthermore, the antigenic peptide from the COOH-terminus of cdc2 did not elute E2F-binding proteins from anti-cdk2 immunoprecipitates (Fig. 2A). Also, reimmunoprecipitation of the material released from the anti-cdk2 immunoprecipitates with anti–cyclin A abolished the band shifts, confirming that cyclin A and cdk2 are part of the same E2F complexes (*30*).

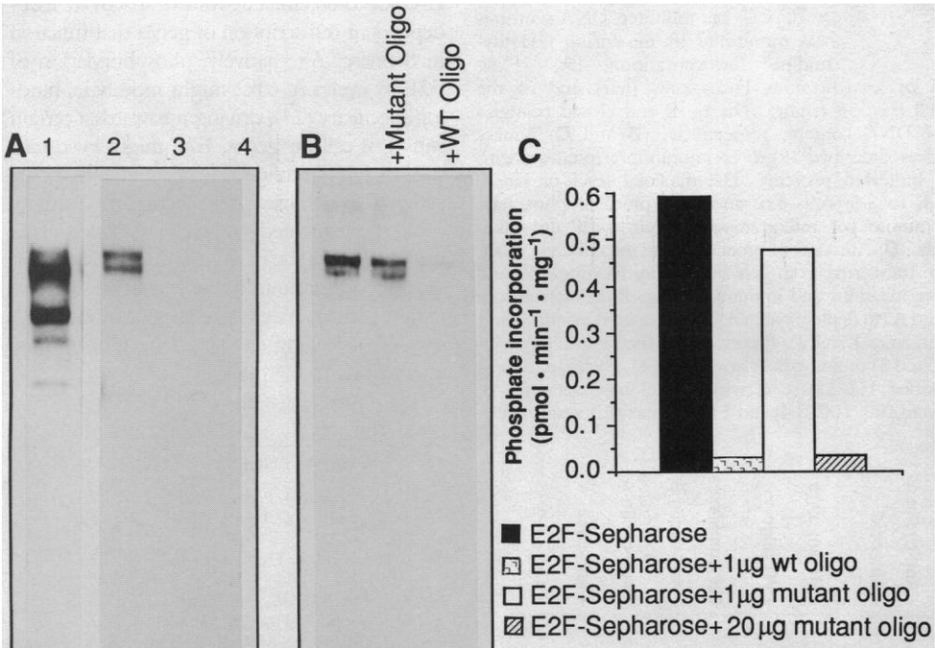


Fig. 2. Binding of cdk2 kinase to E2F. (**A**) HeLa extract (5 mg) was immunoprecipitated with anti-cdk2 or anti-cdc2 as described (*4, 28*). Briefly, cell extracts were prepared by addition of 3 to 5 volumes of lysis buffer B [50 mM tris-HCl (pH 7.4), 0.25 M NaCl, Triton X-100 (0.1%), 5 mM EDTA, 50 mM NaF, 1 mM DTT, and 0.1 mM Na_3VO_4] to a cell pellet. Elution was performed by incubating the pellets with COOH-terminus peptide (250 μg/25 μl) for 3 hours on ice with occasional mixing. The material eluted from an anti-cdk2 immunoprecipitation with cdk2 peptide (lane 2), from an anti-cdc2 immunoprecipitation with cdc2 peptide (lane 3), or from an anti-cdk2 immunoprecipitation with cdc2 peptide (as a control) (lane 4) was then assayed for E2F-binding activity. E2F complexes obtained as in Fig. 1 (lane 1) (Fuji film, overnight exposure) are compared to the eluted material assayed for E2F binding (lanes 2 to 4) (Kodak film, 3-day exposure). (**B**) Binding specificity of the material eluted from anti-cdk2 immunoprecipitations was assessed by competition with the wild-type or mutant oligonucleotide. (**C**) Histone H1 kinase activity associated with E2F. HeLa extract (0.5 mg) was incubated with herring sperm DNA (100 μg) with or without mutant or wild-type oligonucleotide for 15 min on ice. E2F-Sepharose (50 μl) (30 μg of wild-type oligo per milliliter of wet gel) (*38*) was used for each precipitation. Kinase reactions were performed as described (*9*). Briefly, after immunoprecipitation, samples (50 μl) were incubated at 30°C in the presence of 50 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 40 μM ATP, 25 μCi of [γ-^{32}P]ATP and histone H1 (0.2 mg/ml) for 5 min. Nonspecific activity (obtained in the presence of 200 μg wild-type oligonucleotide) was subtracted from each sample.

To test whether an active histone H1 kinase was bound to E2F-complexes, we incubated a HeLa cell extract with E2F-oligo–Sepharose prepared by coupling multimers of the E2F binding site to activated Sepharose and then assayed bound proteins for kinase activity. We detected a histone kinase activity that appeared to be specifically bound to E2F complexes because its binding to the column was prevented by the addition of wild-type oligonucleotide but not mutant oligonucleotide (much higher amounts of the mutant oligonucleotide, sufficient to inhibit the band shift, were required to displace the kinase) (Fig. 2C). The detected kinase activity was a small fraction of the total histone H1 kinase in the cell, but this was expected given the very low abundance of E2F (*31*).

The amount of the cdk2 protein, like that of the cdc2 protein, was constant in cycling cells, whereas cyclin A and cyclin B were absent in G1 cells and their amounts slowly increased as cells progressed through the cycle (Fig. 3D). The histone H1 kinase activity associated with cdk2 paralleled the kinase activity associated with cyclin A, although less activity was measured with anti-cdk2 under these conditions (Fig. 3, B and C). In particular, the kinase activity was high in cells in S phase and G2, and it preceded the activation of the kinase activity associated with cyclin B or cdc2. The cdk2 kinase activity was also high in cells arrested with hydroxyurea at the G1-S transition (*32*). Furthermore, complete depletion of cyclin A from cell extracts abolished 90% of the detectable histone H1 kinase associated with cdk2 demonstrating that the cyclin partner of active cdk2 in these cells is mostly cyclin A.

E2F gel retardation assays were done with cultures of cells undergoing synchronous progression through the cell cycle. The specific DNA binding complexes abolished by anti–cyclin A or anti-cdk2 antibodies in asynchronous cells were not present in G1 cells, but they appeared in early S phase cells

and disappeared again as cells progressed towards mitosis (Fig. 4A). In extracts from G1 cells none of the DNA binding complexes could be dissociated by anti–cyclin A or anti-cdk2 (Fig. 4B), but displacement of DNA binding proteins occurred efficiently in extracts from S phase cells (Fig. 4C). This demonstrates that despite the fact that the amount of cdk2 in the cell is constant during the cell cycle, binding of cdk2 to E2F only occurs in S phase cells, probably as a consequence of its association with cyclin A.

Microinjection of human cells in G1 with anti–cyclin A inhibits entry into S phase (*9*). We have shown that in addition to cyclin A, cdk2 is a component of E2F complexes and that the binding of E2F to cdk2 is regulated in the cell cycle in parallel with the appearance of the cdk2–cyclin A kinase activity. An active kinase, probably having cdk2 as its catalytic subunit was detected in E2F complexes. A clear assessment of the functional role of the E2F-cdk2 interaction will probably have to await the cloning of this transcription factor. Cyclin A–cdk2 could either activate or inactivate E2F-dependent transcription of genes that function in S phase. Alternatively, phosphorylation of E2F by cyclin A–cdk2 might modify its binding specificity (*13*), driving it towards a certain subset of cellular genes. E2F might even take part in DNA replication independently of its activity as a transcription factor; this activity could be regulated by cyclin A–cdk2. This study supports the new view that one function of the cyclin subunits of the cdk kinases may be targeting these enzymes to specific intracellular sites (*33*), leading to stable enzyme-substrate interactions.

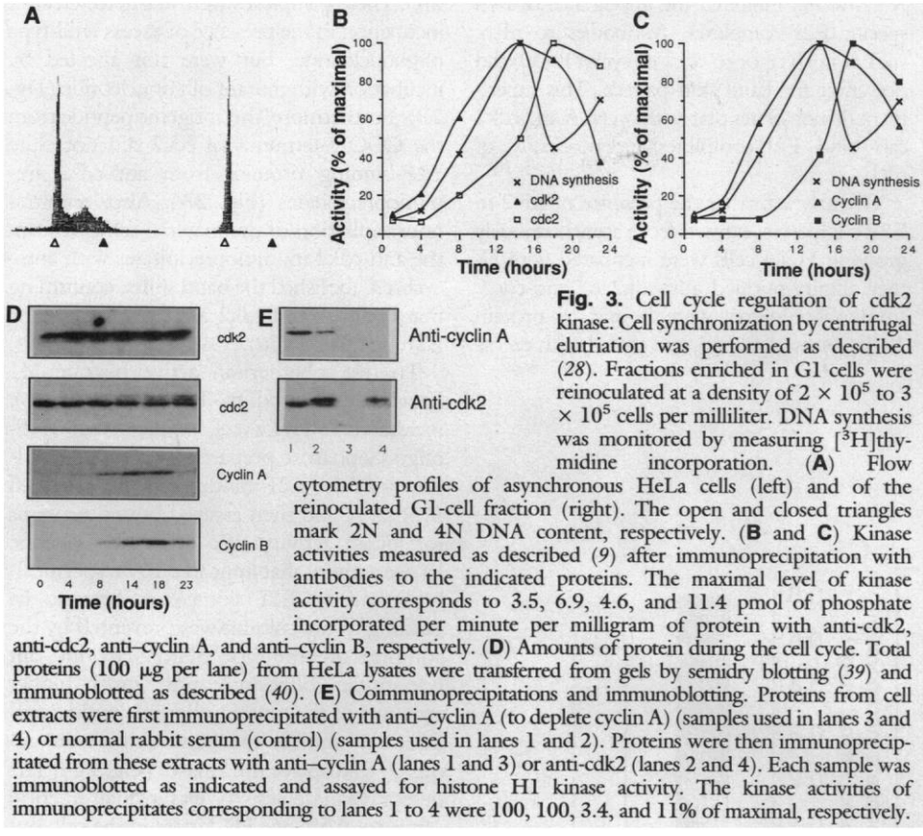


Fig. 3. Cell cycle regulation of cdk2 kinase. Cell synchronization by centrifugal elutriation was performed as described (*28*). Fractions enriched in G1 cells were reinoculated at a density of 2×10^5 to 3×10^5 cells per milliliter. DNA synthesis was monitored by measuring [^{3}H]thymidine incorporation. (**A**) Flow cytometry profiles of asynchronous HeLa cells (left) and of the reinoculated G1-cell fraction (right). The open and closed triangles mark 2N and 4N DNA content, respectively. (**B** and **C**) Kinase activities measured as described (*9*) after immunoprecipitation with antibodies to the indicated proteins. The maximal level of kinase activity corresponds to 3.5, 6.9, 4.6, and 11.4 pmol of phosphate incorporated per minute per milligram of protein with anti-cdk2, anti-cdc2, anti–cyclin A, and anti–cyclin B, respectively. (**D**) Amounts of protein during the cell cycle. Total proteins (100 μg per lane) from HeLa lysates were transferred from gels by semidry blotting (*39*) and immunoblotted as described (*40*). (**E**) Coimmunoprecipitations and immunoblotting. Proteins from cell extracts were first immunoprecipitated with anti–cyclin A (to deplete cyclin A) (samples used in lanes 3 and 4) or normal rabbit serum (control) (samples used in lanes 1 and 2). Proteins were then immunoprecipitated from these extracts with anti–cyclin A (lanes 1 and 3) or anti-cdk2 (lanes 2 and 4). Each sample was immunoblotted as indicated and assayed for histone H1 kinase activity. The kinase activities of immunoprecipitates corresponding to lanes 1 to 4 were 100, 100, 3.4, and 11% of maximal, respectively.

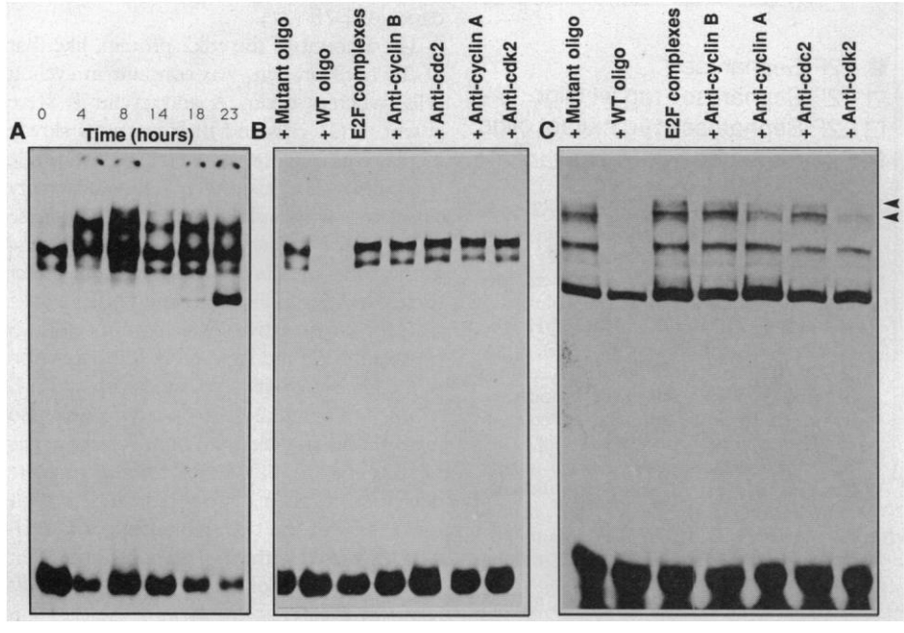


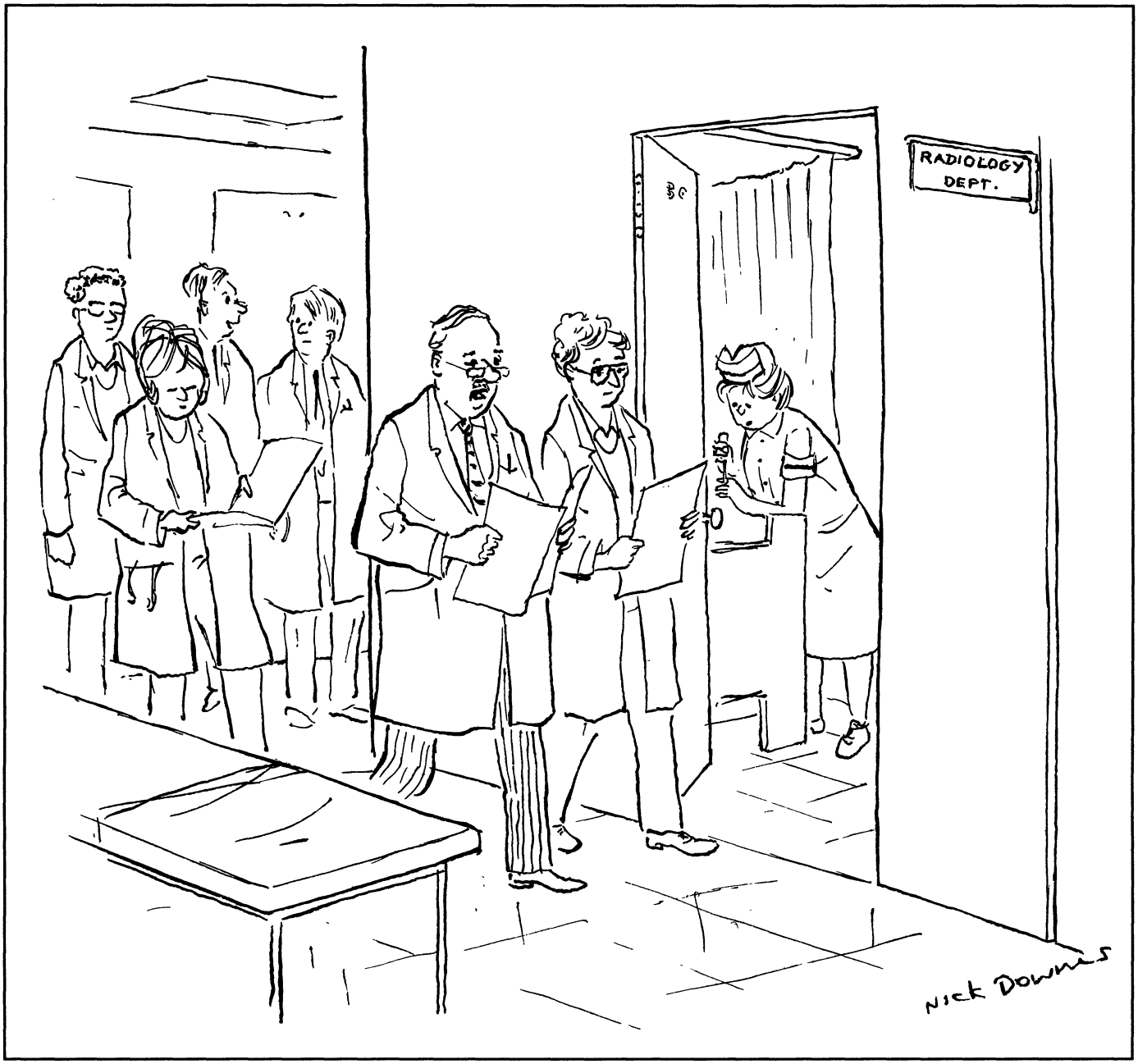
Fig. 4. Cell cycle regulation of E2F complexes. (**A**) Cells from the same experiment described in Fig. 3 were used for E2F band shift assays. (**B**) A G1 phase–enriched fraction (time 0) was assayed in the presence of excess wild-type or mutant oligonucleotide, or the indicated antibodies. (**C**) An S phase–enriched fraction (8 hours after reinoculation) were assayed as in (B). Arrowheads point to the complexes that were less abundant after incubation with both anti-cdk2 and anti–cyclin A antibodies. Affinity-purified antibodies to cyclin B (anti–cyclin B) were obtained by immunizing rabbits with purified recombinant human cyclin B, and purifying the serum on cyclin B–Sepharose (*41*). The bottom band appeared only in some experiments, probably as a result of E2F dissociation.

REFERENCES AND NOTES

1. T. Hunter and J. Pines, *Cell* **66**, 1071 (1991).
2. M. Pagano and G. Draetta, *Prog. Growth Factor Res.*, in press.
3. J. Pines and T. Hunter, *Trends Cell. Biol.* **1**, 117 (1991).
4. A. Giordano *et al.*, *Cell* **58**, 981 (1989).
5. J. Pines and T. Hunter, *Nature* **346**, 760 (1990).
6. A. Giordano, C. McCall, P. Whyte, B. R. Franza, *Oncogene* **6**, 481 (1991).
7. J. Wang, X. Chenivesse, B. Henglein, C. Brechot, *Nature* **343**, 555 (1990).
8. J. Pines and T. Hunter, *J. Cell Biol.* **115**, 1 (1991).
9. M. Pagano, R. Pepperkok, F. Verde, W. Ansorge, G. Draetta, *EMBO J.*, in press.
10. L.-H. Tsai, E. Harlow, M. Meyerson, *Nature* **353**, 174 (1991).
11. A. Giordano *et al.*, *Science* **253**, 1271 (1991).
12. I. Kovesdi, R. Reichel, J. R. Nevins, *Cell* **45**, 219 (1986).
13. S. Bagchi, P. Raychaudhuri, J. R. Nevins, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4352 (1989).
14. ———, *Cell* **62**, 659 (1990).
15. H. Bocuf, B. Reimund, P. Jansen-Durr, C. Kédinger, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1782 (1990).
16. M. C. Blake and J. C. Azizkhan, *Mol. Cell. Biol.* **9**, 4994 (1989).
17. K. Thalmeier, H. Synovzik, R. Mertz, E. L. Winnacker, M. Lipp, *Genes Dev.* **3**, 327 (1989).
18. M. Mudryj, S. W. Hiebert, J. R. Nevins, *EMBO J.* **9**, 2179 (1990).
19. S. W. Hiebert, M. Blake, J. Azizkhan, J. R. Nevins, *J. Virol.* **65**, 3547 (1991).
20. J. Nevins, *Trends Biochem. Sci.* **16**, 435 (1991).
21. B. E. Pearson, H. P. Nasheuer, T. S. F. Wang, *Mol. Cell. Biol.* **11**, 2081 (1991).
22. M. Mudryj *et al.*, *Cell* **65**, 1243 (1991).
23. S. Bagchi, R. Weinmann, P. Raychaudhuri, *ibid.*, p. 1063.

24. S. P. Chellappan, S. Hiebert, M. Mudryi, J. M. Horowitz, J. R. Nevins, *ibid.*, p. 1053.
25. T. Chittenden, D. Livingston, W. G. Kaelin, *ibid.*, p. 1073.
26. P. Raychaudhuri *et al.*, *Genes Dev.* **5**, 1200 (1991).
27. P. Whyte, N. Williamson, E. Harlow, *Cell* **56**, 67 (1989).
28. G. Draetta and D. Beach, *ibid.* **54**, 17 (1988).
29. L. Brizuela, G. Draetta, D. Beach, *EMBO J.* **6**, 3507 (1987).
30. M. Pagano, G. Draetta, P. Jansen-Dürr, unpublished results.
31. A. Yee, P. Raychaudhuri, L. Jakoi, J. R. Nevins, *Mol. Cell. Biol.* **9**, 578 (1989).
32. M. Pagano and G. Draetta, unpublished results.
33. G. Draetta, *Trends. Biochem. Sci.* **15**, 378 (1990).
34. M. Pagano, M. Durst, S. Joswig, G. Draetta, P. Jansen-Dürr, unpublished results.
35. V. Kumar and P. Chambon, *Cell* **55**, 507 (1988).
36. P. Jansen-Durr, H. Boeuf, C. Kedinger, *EMBO J.* **8**, 3365 (1989).
37. P. Whyte *et al.*, *Nature* **334**, 124 (1988).
38. P. Baeuerle and D. Baltimore, *Genes Develop.* **3**, 1681 (1989).
39. E. Harlow and D. Lane, *Antibodies. A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988).
40. G. Draetta, L. Brizuela, J. Potashkin, D. Beach, *Cell* **50**, 319 (1987).
41. V. Baldin and G. Draetta, unpublished results.
42. We thank S. Joswig and M. Stein for technical assistance; E. Harlow and M. Philippe for providing cdk2 clones; G. Smith for helping with flow cytometry; S. Parrella for his contribution to this work; and S. Courtneidge, V. Sorrentino, and G. Superti-Furga for critically reading the manuscript. M.P. was supported by a postdoctoral fellowship from Associazione Italiana per la Ricerca sul Cancro. G.D. was supported in part by NATO grant CGR900652.

2 December 1991; accepted 21 January 1992



"It disturbs me that so many of today's young people go into medicine for the money. In my time, the chance to play God was enough."