Cyclin-Dependent Regulation of G_1 in Mammalian Fibroblasts

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Eukaryotic cells become committed to proliferate during the G_1 phase of the cell cycle. In budding yeast, commitment occurs when the catalytic subunit of a protein kinase, encoded by the *CDC28* gene (the homolog of the fission yeast *cdc2*$^+$ gene), binds to a positively acting regulatory subunit, a cyclin. Related kinases are also required for progression through the G_1 phase in higher eukaryotes. The role of cyclins in controlling G_1 progression in mammalian cells was tested by construction of fibroblasts that constitutively overexpress human cyclin E. This was found to shorten the duration of G_1, decrease cell size, and diminish the serum requirement for the transition from G_1 to S phase. These observations show that cyclin levels can be rate-limiting for G_1 progression in mammalian cells and suggest that cyclin synthesis may be the target of physiological signals that control cell proliferation.

The proliferation of all eukaryotic cells is primarily regulated by a decision that occurs during the G_1 phase of the cell cycle—to remain in the cell cycle and divide or to withdraw from the cell cycle and adopt an alternative cell fate (*1*). In budding yeast this decision, called Start, is the physiological process whereby specific extracellular and intracellular signals combine to promote either cell cycle progression or cell cycle arrest and preparation for conjugation (*2, 3*). Completion of Start, which is followed by the execution of S phase, commits the yeast cell to complete the remainder of the cell division cycle. Proliferation of mammalian fibroblasts also is regulated by mitogenic signals during the G_1 phase of the cell cycle, and cells can switch between quiescence and proliferation at a unique point in G_1 (*1*). Once a cell passes this point it will complete the cell cycle even if mitogenic signals are removed. Thus, transit through the G_1 control point commits the cell to complete the remainder of the cell cycle and in this sense appears physiologically analogous to Start. The factors that regulate proliferation of yeast, however, such as nutrients, mating pheromones, and cell size, contrast with the central role played by mitogenic serum growth factors in multicellular higher eukaryotes (*1, 2, 4*). Thus, a fundamental question is whether biochemical events similar to those occurring at Start also have a prominent role in controlling G_1 progression in higher eukaryotes. Experiments in a number of model systems support the idea that, like yeast, higher eukaryotes require a cyclin-dependent kinase (CDK) for completion of G_1 and the onset of DNA replication (*5*). CDKs are activated as a consequence of binding to a cyclin (*6, 7*), and in budding yeast accumulation of cyclins during G_1 is rate-limiting for execution of Start (*3, 8*). Mutations that stabilize the G_1 cyclins, or overexpression of G_1 cyclins, decrease the duration of G_1 and result in small cell size during exponential growth (*8*). Moreover, at least some factors that regulate Start may do so by controlling the synthesis or activity of the G_1 cyclins (*3, 9*). It is critical, therefore, to determine the extent to which the complex physiological controls on G_1 progression in higher eukaryotes might also be mediated by expression of cyclins. Accordingly, we examined the consequences of constitutively overexpressing a candidate human G_1 cyclin, cyclin E (*10, 11*), in Rat-1 fibroblasts and primary human diploid fibroblasts.

A human cyclin E cDNA was cloned into a retroviral expression vector, LXSN (*12*), that uses the 5′ long terminal repeat of Molony murine sarcoma virus to express the inserted cDNA and contains the neomycin phosphotransferase gene (G418 resistance) as a selectable marker. Rat-1 and primary human foreskin fibroblasts (passage six, designated T-6) were infected with LXSN and LXSN–cyclin E and pools of over ten thousand transformants selected by growth for 2 weeks in G418. For the analyses described below, we generated ten independent pools of Rat-1 cells and six independent pools of primary human fibroblasts infected with LXSN and LXSN–cyclin E. All experiments were performed on at least two independent pools and usually on all pools.

At least two forms of the endogenous cyclin E protein were detected in exponentially proliferating control fibroblasts, at 50 and 55 kD (Fig. 1A). The 50-kD form is maximally expressed during late G_1 and early S phase (*10*) and is induced during mitogenic activation of primary human T lymphocytes (*13*). Cells infected with LXSN–cyclin E exhibited increased expression of only the 50-kD cyclin E protein. Overexpression of the 50-kD cyclin E protein suppressed expression of the endogenous 55-kD form. The 50-kD overexpressed protein corresponded to the predicted mo-

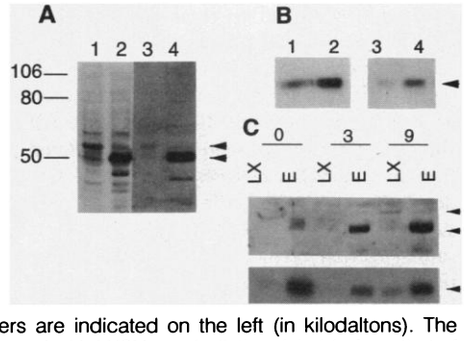

Fig. 1. Constitutive overexpression of human cyclin E in mammalian fibroblasts. (**A**) Expression of human cyclin E. We determined the presence of cyclin E in total protein from 2 × 10^5 cells by immunoblotting with affinity-purified antibodies to cyclin E (*10*). Immunoreactive proteins were detected by enhanced chemiluminescence (ECL) (Amersham). Exponentially growing Rat-1 fibroblasts (lanes 1 and 2) and primary human foreskin fibroblasts (lanes 3 and 4) were infected with LXSN (lanes 1 and 3) or LXSN–cyclin E (lanes 2 and 4). Positions of two different forms of cyclin E are indicated by arrowheads. Molecular size markers are indicated on the left (in kilodaltons). The expression and activity of cyclin E in cells transfected with LXSN was indistinguishable from that of cells that had not been transfected. (**B**) Cyclin E–associated histone H1 kinase activity. Lysates (200 μl) from 2 × 10^6 cells were incubated with rabbit antiserum specific to cyclin E (2 μl) for 60 min on ice. Immunoprecipitates were collected on protein A–Sepharose, washed, and tested for H1 kinase activity (*10*). Exponentially growing Rat-1 fibroblasts (lanes 1 and 2) and human fibroblasts (lanes 3 and 4) were infected with LXSN (lanes 1 and 3) or LXSN–cyclin E (lanes 2 and 4). The position of histone H1 is indicated by an arrowhead. (**C**) Expression and activity of cyclin E during the cell cycle. Exponentially growing Rat-1 fibroblasts infected with LXSN (LX) and LXSN–cyclin E (E) were treated with Nocodazole (0.1 μg/ml) (*15*) for 5 hours, and mitotic cells were collected by gentle pipetting. More than 98% of the harvested cells were mitotic. Cells were washed with Dulbecco's modified Eagle's medium (DMEM) and plated at 5 × 10^5 cells per 100-mm dish with DMEM plus calf serum (10%). Samples were prepared after 0, 3, or 9 hours, as indicated, for both protein immunoblotting and assays of cyclin E–associated H1 kinase activity. Total protein from to 2 × 10^5 cells before release was used for protein immunoblotting, and lysates from 5 × 10^5 cells were used for the H1 kinase assay. Positions of cyclin E proteins (upper panel) and phosphorylated histone H1 (lower panel) are indicated by arrowheads.

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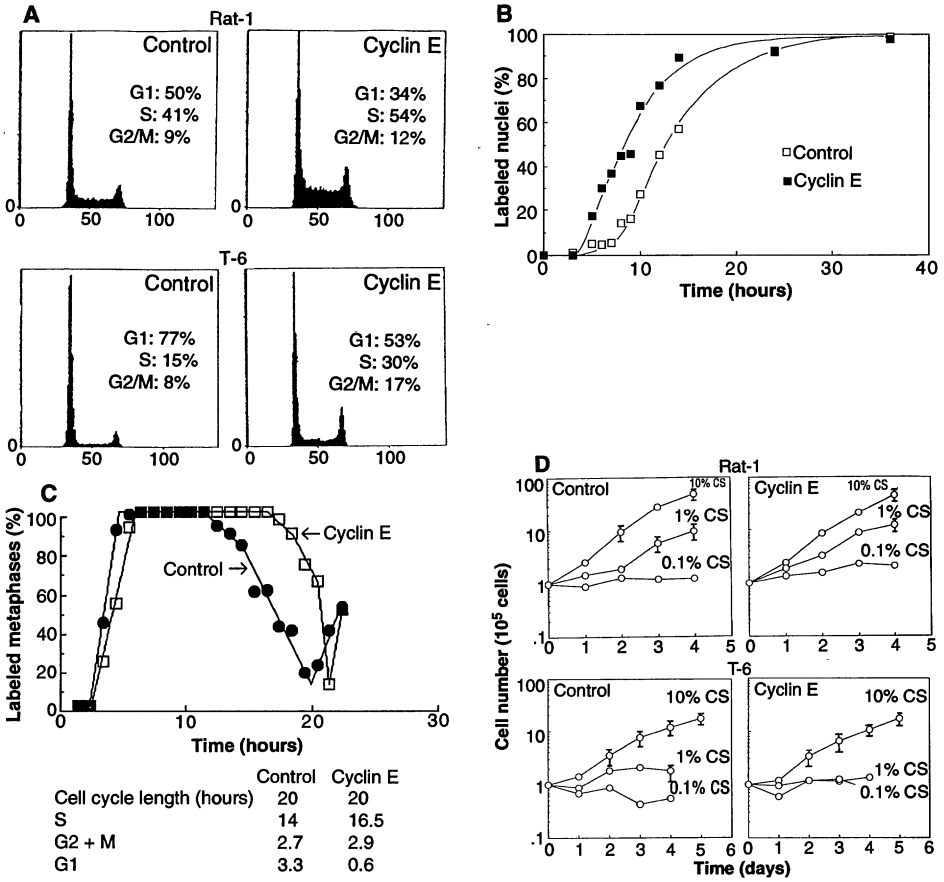
lecular size of human cyclin E and comigrated with human cyclin E produced from the same cDNA by a baculoviral vector (*14*). In many experiments, overexpression of a 40-kD protein was also observed. This may have resulted from proteolytic degradation of the overexpressed cyclin E or from translation initiation at an internal methionine residue. Overexpression of the cyclin E protein resulted in a three- to fivefold increase in the amount of cyclin E–associated histone H1 kinase activity in exponentially growing Rat-1 fibroblasts and human fibroblasts (Fig. 1B).

In control cells, activity of the cyclin E–associated kinase was cell cycle–dependent (Fig. 1C). The three- to fivefold fluctuation in cyclin E–associated kinase activity during the cell cycle of Rat-1 cells was similar to that observed in MANCA cells, a human lymphoblastoid cell line (*10*). In cells infected with LXSN–cyclin E, the cyclin E–associated kinase was relatively independent of the cell cycle; there was a high kinase activity in metaphase cells that persisted in G_1 and S phase cells. In both control cells and cells infected with LXSN–cyclin E, the amount of cyclin E protein reflected the amount of cyclin E–associated kinase activity (Fig. 1C), with one possible exception. In metaphase cells, relatively less cyclin E protein was detected, but the cyclin E–associated kinase activity was high. The cyclin E protein in these cells showed a slightly decreased electrophoretic mobility and appeared more heterogeneous in molecular size, features that might be indicative of posttranslational modification. At 3 hours after release from metaphase arrest (*15*), cells constitutively expressing cyclin E contained high amounts of cyclin E–associated kinase activity; however, S phase did not begin for another 2 to 3 hours (Fig. 2B). Therefore, increased amounts of cyclin E and cyclin E–associated kinase were not sufficient to initiate DNA replication.

We determined the effect of constitutive cyclin E expression on the distribution of cells in the cell cycle (Fig. 2A). Cell populations constitutively expressing cyclin E showed a decreased number of cells in G_1 and an increased number in S phase. This change in cell cycle distribution is consistent with accelerated transit through G_1 and is similar to the phenotype resulting from moderate overexpression of G_1 cyclins in budding yeast (8). We used two methods to demonstrate directly that this change in cell cycle distribution was due, in part, to a decrease in the duration of G_1. We measured the time between mitosis and the start of S phase in synchronized Rat-1 cells (Fig. 2B). In four separate experiments, the duration of G_1 was on average 3 hours (33%) shorter in cells infected with LXSN–cyclin E than in control cells. Analogous experiments with T-6 human fibroblasts also showed that the duration of G_1 in cells infected with LXSN–cyclin E was about 4 hours shorter than in control cells. We also used the method of labeled mitoses (*16*) (Fig. 2C), which determines the length of each phase of the cell cycle in asynchronous, proliferating cell populations without the use of synchronizing agents. This con-



	Control	Cyclin E
Cell cycle length (hours)	20	20
S	14	16.5
G2 + M	2.7	2.9
G1	3.3	0.6

Fig. 2. Effect of constitutive cyclin E expression on cell cycle kinetics. (**A**) Flow cytometric analysis. Cells infected with LXSN (Control) and LXSN–cyclin E (Cyclin E) were plated at 5×10^5 cells per 100-mm dish with calf serum (10%). Two days later, cells were collected by trypsinization, fixed, and stained. Cells (10^6) in a propidium iodide solution (0.5 ml) were analyzed by FACScan with LYSYS II software (Becton Dickinson). The percentage of cells in each phase of the cell cycle was determined with Multicycle software (Phoenix Flow Systems). Histograms show relative DNA content (*x*-axis) and cell number (*y*-axis). Flow-cytometric analysis was performed on ten independent pools of Rat-1 cells and six independent pools of primary human fibroblasts infected with LXSN and LXSN–cyclin E. Fifty-five independent analyses were performed, and results similar to those depicted were observed 41 times (41/55 = 75%). In no case did the control cells show a decline in the G_1 fraction relative to the cells overexpressing cyclin E (0/51 = 0%). (**B**) Length of G_1 in synchronized cells. Mitotic cells were prepared as in Fig. 1C and plated at 5×10^4 cells per 35-mm dish in DMEM containing calf serum (10%). Bromodeoxyuridine (BrdU) was added at 0 hours, and samples were stained with peroxidase-conjugated antibodies to BrdU (Amersham) at each time point. The percentage of cells that were labeled was plotted versus time. At least 500 cells were counted at each time point. At time points corresponding to early S phase, the labeling intensity of cells overexpressing cyclin E was noticeably lower than control cells. This may have reflected a slower rate of progression through S phase (Fig. 2C). (**C**) Frequency of labeled mitoses. Asynchronous Rat-1 cells were labeled with tritiated thymidine for 30 min, and the percentage of labeled metaphases was scored at regular intervals (*16*). The percentage of metaphases labeled by tritiated thymidine were plotted versus time. One hundred metaphase cells were counted at each time point. Four hundred metaphase cells were counted at the final time point. At the two hour time point, the labeling index was 54% for control cells and 68% for cells overexpressing cyclin E. The mitotic index was 3% for both cell populations. Numerous factors probably contributed to the differences in the G_1 lengths as measured by the methods described in Fig. 2, A to C (*30*). (**D**) Population doubling times. Triplicate cultures of control cells and cells infected with LXSN–cyclin E were cultured in DMEM containing the indicated concentrations of calf serum; live cells were counted at daily intervals. Mean values of the triplicates with standard errors are plotted versus time. The population doubling times were 18 hours for Rat-1 fibroblasts and 26.4 hours for human fibroblasts growing in 10% serum. Overexpression of cyclin E had no effect on population doubling times.

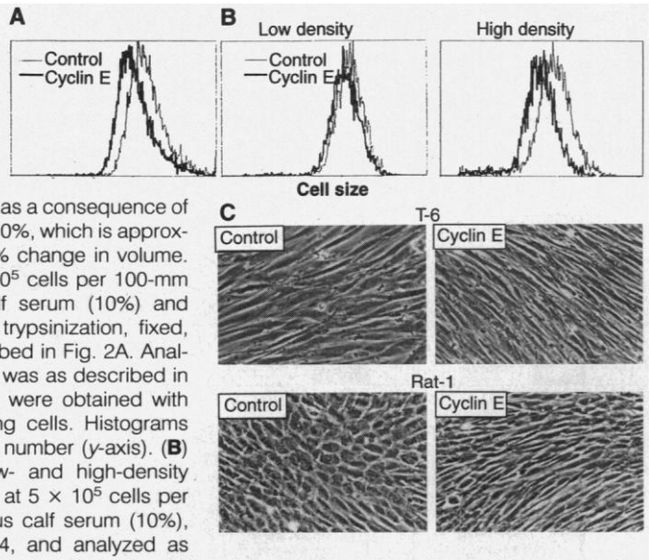


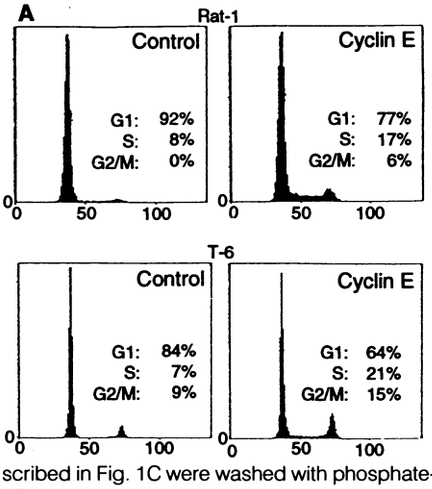
Fig. 3. Effect of constitutive expression of cyclin E on cell size. (**A**) Measurement of cell size in exponentially proliferating human fibroblasts. FSC (size) for the G_1 cell population is shown. The relative change in diameter as a consequence of cyclin E overexpression is 10%, which is approximately equivalent to a 30% change in volume. Cells were plated at 5×10^5 cells per 100-mm dish with DMEM plus calf serum (10%) and collected 2 days later by trypsinization, fixed, and then stained as described in Fig. 2A. Analysis by FACScan (for FSC) was as described in Fig. 2A. Equivalent results were obtained with either ethanol-fixed or living cells. Histograms show FSC (*x*-axis) and cell number (*y*-axis). (**B**) Size of Rat-1 cells in low- and high-density cultures. Cells were plated at 5×10^5 cells per 100-mm dish in DMEM plus calf serum (10%), collected at days 2 and 4, and analyzed as described. The cultures were almost confluent after 4 days and were less than 50% confluent after 2 days. The difference in cell size between control and cyclin E–expressing cells was determined for samples after 2 days (low density) and 4 days (high density). (**C**) Control and cyclin E–expressing cells were plated at 5×10^5 cells per 100-mm dish with DMEM containing calf serum (10%); after 4 days (Rat-1 fibroblasts) or 7 days (human fibroblasts), photomicrographs were taken. Magnification, ×160.

firmed that cells constitutively expressing cyclin E had a G_1 considerably shorter than that of control cells and, therefore, implied that cyclin accumulation can be rate-limiting for transit through G_1. This analysis also showed that the total length of the cell cycle was not changed by overexpression of cyclin E (Fig. 2C). This was attributable to a shortening of G_1 in combination with a lengthening of both S and G_2 relative to control cells. We found that, consistent with these observations, the population doubling time of control Rat-1 cells and T-6 human fibroblasts was very similar to that of cells constitutively expressing cyclin E (Fig. 2D).

These observations raise questions about the cause and effect relation between the shortened G_1 and the lengthened S + G_2 in cells overexpressing cyclin E. We consider it most likely that the primary effect of cyclin E overexpression was to shorten G_1 and suggest that, as a consequence, cells entered S phase with suboptimal amounts of one or more factors that are necessary for bringing about DNA replication and mitosis at normal rates. Overexpression of G_1 cyclins in budding yeast also appears to cause secondary delays in S or G_2 (*17*). On the other hand, the primary effect of cyclin E may be to delay S and G_2, possibly by inhibiting the S and G_2 cyclins. Thus, the shortened G_1 could have been a consequence of the increased time available for cell growth in the previous cell cycle (*1, 18, 19*). The results of other experiments indicated that this latter mechanism is unlikely to explain the shortened G_1: Overexpression of cyclin E decreased cell size throughout the cell cycle, which is inconsistent with the idea that alteration in G_1 length may be a secondary consequence of an extended growth period in S and G_2. Also, overexpression of cyclin E altered the serum requirement for the G_1 to S transition, which indicates a direct effect of cyclin E on G_1 control.

Both budding and fission yeast coordinate cell growth with cell division by restricting the frequency of DNA replication to approximately once per mass doubling (*2, 19*). This is accomplished by the requirement that cells attain a critical size in order to complete Start. Yeast appear to use the amount of the G_1 cyclins as a molecular measure of cell size because CLN (G_1 cyclin) overexpression promotes early entry into S phase and decreases cell size (*3, 8*). Therefore, we determined the effect of cells overexpressing cyclin E on the size of mammalian fibroblasts. We used the flow cytometric parameter of forward angle light scatter (FSC) to measure cell volume (*20*) and found that constitutive expression of cyclin E caused primary human fibroblasts to exit G_1 with a volume that was 30% smaller than that of control cells (Fig. 3A). Although cells overexpressing cyclin E were smaller than control cells at all stages of the cell cycle, the relative effect was greater in G_1 cells than in G_2 cells. Furthermore, the amount of protein per cell in an asynchronous population was decreased by about 30% (*21*). Cells overexpressing cyclin E were smaller than control cells even though the population doubling time for the two cell types was the same. This implies that fibroblasts have a mechanism for adjusting cell size that is related to the level of G_1 cyclins and that cell size is not simply a correlate of cell cycle length.

During exponential growth, Rat-1 cells overexpressing cyclin E were 10 to 25% smaller than control cells; these differences were not as large as those observed with primary human fibroblasts. At high cell densities, however, overexpression of cyclin E typically caused a 30% decrease in volume relative to control cells (Fig. 3, B and C). The volume of control Rat-1 fibroblasts also diminished as the cell density increased. This may reflect increased competition for nutrients or serum growth factors, or both. An analogous phenomenon has been described in budding and fission yeast, in which a decrease in the critical cell size for completion of Start occurs in response to nutrient limitation (*19*). Our



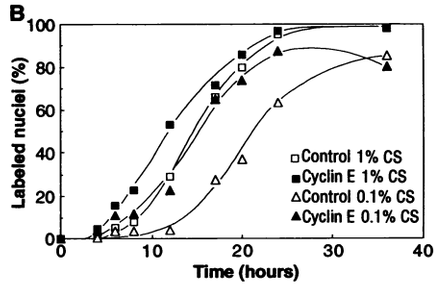


Fig. 4. Effect of constitutive expression of cyclin E on cell growth in low concentrations of serum. (**A**) DNA content. Cells plated at 5×10^5 cells per 100-mm dish with DMEM containing 0.1% calf serum were collected 2 days later and analyzed by FACScan as described in Fig. 2A. (**B**) G_1 length. Mitotic cells prepared as described in Fig. 1C were washed with phosphate-buffered saline and then plated at 5×10^4 cells per 35-mm dish in DMEM containing calf serum (1 or 0.1%). G_1 length was analyzed as described in Fig. 2B. At least 500 cells were counted at each time point.

data suggest that this response was exaggerated in Rat-1 cells overexpressing cyclin E. The saturation density of Rat-1 and human fibroblasts overexpressing cyclin E was 30 to 40% greater than that of control cells (Fig. 3C). This change may be related to the effect of cyclin E on cell size and, to a lesser degree, the ability of these cells to continue to proliferate in diminished levels of serum growth factors.

To test whether the mitogenic effects of serum growth factors are mediated, at least in part, by cyclin accumulation, we determined the effect of constitutive cyclin E expression on cell proliferation that occurred in the presence of decreased concentrations of serum. Cells growing in medium supplemented with 10% calf serum were transferred to 0.1% calf serum for 48 hours, and the distribution of cells within the cell cycle was measured by flow cytometry (Fig. 4A). After 48 hours in 0.1% serum, both Rat-1 and primary human fibroblasts exhibited a large decrease in the percentage of cells in S phase and an increase in the fraction of cells in G_1, relative to cells grown in 10% serum (Figs. 2A and 4A). In contrast, overexpression of cyclin E resulted in a smaller decline in the percentage of cells in S phase in response to low serum concentrations. The length of G_1 was then measured in the first cell cycle after Rat-1 cells were transferred from 10 to 1 or 0.1% serum. Cells constitutively expressing cyclin E showed a 6-hour increase in the median length of G_1 (the point at which 50% of nuclei are labeled) when switched from 10 to 0.1% serum—from 8 to 14 hours (Fig. 4B). In contrast, the median length of G_1 for the control cells increased by 12 hours—from 11 hours in 10% serum to 23 hours in 0.1% serum. Taken together, these experiments show that overexpression of cyclin E partially reversed the delay in G_1 imposed in the first cell cycle after serum withdrawal and suggest that cyclin accumulation, under these conditions, might be a limiting event controlled by serum growth factors.

Although cells overexpressing cyclin E showed a reduced delay in the first cell cycle after serum withdrawal, they did not continue to proliferate. As the concentration of serum declined, the population doubling time for control cells and cells overexpressing cyclin E declined equivalently, and in 0.1% serum essentially no population doublings occurred (Fig. 2D). Therefore, although a number of parameters related to cell cycle regulation are altered, there are a number of reasons why cells overexpressing cyclin E are not transformed: They require serum for prolonged proliferation, they do not form foci or grow in soft agar (*22*), and primary human fibroblasts expressing cyclin E are not immortalized (*23*). An increased amount of cyclin E may initially suppress the serum requirement for G_1 transit by constitutively activating a rate-limiting serum-dependent event. However, it may be insufficient to maintain requisite amounts of other essential proteins whose expression remains serum-dependent.

In addition to the physiological observations described above, biochemical evidence also supports a role for cyclin E in G_1 transit (*10, 11, 24, 25*). However, the ability of elevated and constitutive amounts of cyclin E to alter G_1 regulation does not necessarily imply that cyclin E normally functions as a G_1 cyclin. Constitutive expression of some mitotic cyclins can complement mutations in the budding yeast G_1 cyclins (*10, 11, 26*), suggesting that overexpression may override the specificity of cyclin function. We have infected Rat-1 and human T-6 fibroblasts with retroviruses containing cyclins A and B and did not observe phenotypic alterations characteristic of cells infected with cyclin E. These experiments may not be directly comparable because, unlike those with cyclin E, we could not reproducibly express cyclins A and B in amounts greater than those of their endogenous counterparts. Cyclins D1 and D2 have been overexpressed in a mouse macrophage cell line cell (BAC1.2F5) and do not shorten G_1 or advance commitment to S phase (*27*). Therefore, among the cyclins known to be synthesized during G_1, only cyclin E expression has been shown to effect cell cycle kinetics. Our experiments also did not address whether cyclin E is necessary for completing G_1. Budding yeast cells contain at least three G_1 cyclins that, to a large degree, are functionally redundant. Human cyclins A, D, E (*10, 28*), and perhaps C (*11*) are all synthesized during G_1, and the possibility of functional overlap between these cyclins has not been addressed. Furthermore, our experiments have focused on progression from mitosis to S phase and have not investigated whether the rate of transition from quiescence to S phase is similarly limited by cyclin abundance.

In conclusion, our experiments showed that cyclin levels can control the rate of progression through the G_1 phase of the mammalian cell cycle, both in standard growth conditions and in conditions where the rate of the cell cycle is restricted by the level of serum growth factors. Although many proteins are necessary for entry into S phase, accumulation of only two proteins, Myc (*29*) and cyclin E, have been shown to determine the rate of G_1 transit. We conclude, therefore, that cyclin accumulation may be one of the few rate-limiting events in G_1 progression in mammalian cells, and that factors that control commitment to the cell cycle, such as those present in serum, may do so by controlling cyclin activity.

REFERENCES AND NOTES

1. A. B. Pardee, *Science* **246**, 603 (1989); F. Cross, J. Roberts, H. Weintraub, *Annu. Rev. Cell Biol.* **5**, 341 (1989); C. Norbury and P. Nurse, *Annu. Rev. Biochem.* **61**, 441 (1992); A. Pardee, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1286 (1974); A. Zetterberg and O. Larson, *ibid.* **82**, 5365 (1985); W. Pledger, C. Stiles, H. Antoniades, C. Scher, *ibid.* **75**, 2389 (1978).
2. L. Hartwell *et al.*, *Genetics* **74**, 267 (1973); J. R. Pringle and L. H. Hartwell, in *Molecular Biology of the Yeast* Saccharomyces, J. N. Strathern, E. W. Jones, J. R. Broach, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1981), pp. 97–142; L. H. Hartwell, J. Culotti, J. R. Pringle, B. J. Reid, *Science* **183**, 46 (1974).
3. S. I. Reed, *Trends Genet.* **7**, 95 (1991).
4. A. B. Pardee, R. Dubrow, J. L. Hamlin, R. F. Kletzien, *Annu. Rev. Biochem.* **47**, 715 (1978); L. Cantley *et al.*, *Cell* **64**, 281 (1991); M. Chao, *ibid.* **68**, 995 (1992).
5. G. D'Urso, R. L. Marraccino, D. R. Marshak, J. M. Roberts, *Science* **250**, 786 (1990); J. J. Blow and P. Nurse, *Cell* **62**, 855 (1990); F. Fang and J. Newport, *ibid.* **66**, 731 (1991); Y. Furukawa, H. Piwnica-Worms, T. J. Ernst, Y. Kanakura, J. D. Griffin, *Science* **250**, 805 (1990).
6. T. Hunt, *Curr. Opin. Cell Biol.* 1, 286 (1989); J. Pines and T. Hunter, *New Biol.* **2**, 289 (1990).
7. R. Booher and D. Beach, *EMBO J.* **6**, 3441 (1987); M. Soloman, M. Glotzer, T. Lee, M. Phillippe, M. Kirschner, *Cell* **63**, 1013 (1990).
8. F. R. Cross, *Mol. Cell. Biol.* **8**, 4675 (1988); R. Nash, G. Tokiwa, S. Anand, K. Erickson, A. B. Futcher, *EMBO J.* **7**, 4335 (1988); J. A. Hadwiger, C. Wittenberg, H. E. Richardson, M. D. Lopes, S. I. Reed, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6255 (1989); H. Richardson, C. Wittenberg, F. Cross, S. Reed, *Cell* **59**, 1127 (1989); P. E. Sudbery, A. R. Goodey, B. L. Carter, *Nature* **288**, 401 (1980); C. Wittenberg, K. Sugimoto, S. I. Reed, *Cell* **62**, 225 (1990).
9. F. Chang and I. Herskowitz, *Cell* **63**, 999 (1990).
10. A. Koff *et al.*, *ibid.* **66**, 1217 (1991); A. Koff *et al.*, *Science* **257**, 1689 (1992); V. Dulic, E. Lees, S. I. Reed, *ibid.* **257**, 1958 (1992).
11. D. J. Lew, V. Dulic, S. I. Reed, *Cell* **66**, 1197 (1991).
12. The retroviral vector containing cyclin E (LXSN–cyclin E) was constructed by insertion of a blunt-ended Hind III fragment of the human cyclin E cDNA HU4 (*10*) (which contains the entire open reading frame) into the Hpa I site of LXSN, a murine retrovirus–based vector in the sense orientation. Rat-1 and human fibroblasts cells that constitutively expressed cyclin E were produced as described [A. D. Miller and G. J. Rosman, *BioTechniques* **7**, 980 (1989); R. A. Hock, A. D. Miller, W. R. A. Osborne, *Blood* **74**, 876 (1989)]. Cell lines producing virus were analyzed by Southern (DNA) blot analysis and assayed for vector titer. Rat-1 fibroblasts were infected with the ecotropic retrovirus stock, and primary human foreskin fibroblasts (passage 6) were infected with an amphotropic stock. In all experiments, we studied pools of transformants rather than cell clones to avoid any effects that could be attributed to clonal variation within the Rat-1 or human fibroblast populations. Moreover, each infected cell population was grown in continuous culture for no more than 2 months before being replaced with a frozen stock or freshly infected cells.
13. E. J. Firpo, A. Koff, J. Roberts, in preparation
14. M. Ohtsubo and J. M. Roberts, unpublished data.
15. G. W. Zieve, D. Turnbull, M. J. Mullins, R. J. McIntosh, *Exp. Cell Res.* **126**, 397 (1980).
16. Rat-1 fibroblasts were plated at 4×10^4 cells per 35-mm dish in DMEM plus calf serum (10%). Two days later, the cultures were labeled for 15 min with [^{3}H]thymidine (1 μCi/ml, 80 Ci/mmol; Amersham). After labeling, the cultures were washed twice with culture medium and fresh medium supplemented with unlabeled thymidine (100 μM). At each time point after washing was completed,

cells were collected and immediately fixed by exposure to methanol and acetic acid (3:1, v/v). Slides containing fixed cells were air-dried and dipped in NTB2 Kodak emulsion as described by the manufacturer. Dipped slides were air-dried, stored at 4°C for 3 weeks, and then developed and stained with Giemsa. Cell cycle parameters were determined as follows. The time taken to first attain 50% of labeled metaphases is equivalent to G2 + M. The length of time from that point to the next point, where only 50% of metaphases are labeled, is equivalent to the duration of the S phase. The total length of the cell cycle is the interval between any two equivalent points on the curve. G_1 length is obtained by subtraction. [H. Quastler and F. G. Sherman, *Exp. Cell Res.* **17**, 420 (1959); A. Macieira-Coelho, in *Tissue Culture Methods and Applications*, P. F. Krause and M. K. Patterson, Jr., Eds. (Academic Press, New York, 1973), pp. 412–422].

17. F. R. Cross, personal communication.
18. G. Stancel, D. Prescott, M. Liskay, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 6295 (1978).
19. P. Nurse, *Nature* **256**, 457 (1975); P. Fantes and P. Nurse, *Exp. Cell Res.* **107**, 377 (1977); P. Nurse and P. Thuriaux, *ibid.*, p. 365; G. Johnston, J. Pringle, L. Hartwell, *ibid.* **105**, 79 (1977); K. Nasmyth, P. Nurse, R. S. S. Fraser, *J. Cell Sci.* **39**, 215 (1979); K. A. Nasmyth, *ibid.* **36**, 155 (1979).
20. Empirical measurements with calibrated spheres of known dimensions showed that FSC as measured by flow cytometry on a fluorescence-activated cell sorter (FACScan, Becton Dickinson) was linearly related to the diameter of the spheres (J. Roberts, unpublished observations).
21. Subconfluent, proliferating cells were collected by trypsinization, washed twice with phosphate-buffered saline, and suspended in modified Lowry reagent [G. L. Peterson, *Anal. Biochem.* **83**, 346 (1977); V. I. Oyama and H. Eagle, *Proc. Soc. Exp. Biol. Med.* **91**, 305 (1956)]. Protein amount was measured with a protein assay kit (Sigma); bovine serum albumin was used as a standard. Mean values of triplicate cultures were 327.6 ± 6.8 pg of protein per cell for control T-6 human fibroblasts and 243.9 ± 22.0 pg of protein per cell for cells constitutively expressing cyclin E.
22. Rat-1 fibroblasts were infected with retroviral expression vectors (LXSN) encoding cyclin E, c-Myc, and bcr-Abl and grown to confluence. No foci of proliferating cells were detected in cells infected with vectors encoding cyclin E alone or cyclin E in combination with either of the other two proteins. Also, none of these combinations was sufficient to allow growth in soft agar. The combination of c-Myc plus bcr-Abl, however, did induce the formation of foci and soft agar growth (E. Blackwood and M. Ohtsubo, unpublished data).
23. Late-passage primary human diploid fibroblasts were infected with LXSN or LXSN–cyclin E. Cells were selected for resistance to G418 and propagated. Control and cyclin E–overexpressing cells both senesced after 65 population doublings, with no alterations in proliferative potential (T. Norwood, M. Ohtsubo, J. Roberts, unpublished observations).
24. P. W. Hinds *et al.*, *Cell* **70**, 993 (1992).
25. E. Lees, B. Faha, V. Dulic, S. I. Reed, E. Harlow, *Genes Dev.* **6**, 1874 (1992).
26. Y. Xiong, T. Connolly, B. Futcher, D. Beach, *Cell* **65**, 691 (1991).
27. C. Sherr, personal communication.
28. R. L. Marraccino, E. J. Firpo, J. M. Roberts, *Mol. Biol. Cell* **3**, 389 (1992); H. Matsushime, M. F. Roussel, R. A. Ashmun, C. J. Sherr, *Cell* **65**, 701 (1991); J. Pines and T. Hunter, *Nature* **346**, 760 (1990).
29. J. Karn, J. V. Watson, A. D. Lowe, S. M. Green, W. Vedeckis, *Oncogene* **4**, 773 (1989).
30. All experiments were internally consistent because overexpression of cyclin E always shortened G_1 relative to control cells. However, the length of G_1 was different when measured by the three methods described in Fig. 2, A to C. This appears to be the result of small but systematic errors specific to each type of analysis. Analysis by flow cytometry overestimated the percentage of G_1 cells because early S phase cells do not increase their DNA content sufficiently to be distinguished from G_1 cells. This might have had a greater effect for the cells overexpressing cyclin E because they traversed S phase more slowly and therefore accumulated DNA at a slower rate. This was evident in the labeling patterns obtained with tritium and BrdU in which, during early S phase, the intensity of labeling for the control cells was greater than for the cells overexpressing cyclin E. The absolute length of G_1 measured in synchronized cells was also overestimated, owing to the presence of a recovery period after release from Nocodazole-induced mitotic arrest. In contrast, the analysis with labeled mitoses probably underestimated G_1 length. In this method, S and G2/M lengths are most accurately measured. G_1 length is measured indirectly and depends on data derived from time points where the synchrony of the cell populations is declining. Therefore, the result tends to be biased toward the most rapidly proliferating cells.
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NF-κB Controls Expression of Inhibitor IκBα: Evidence for an Inducible Autoregulatory Pathway

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The eukaryotic transcription factor nuclear factor–kappa B (NF-κB) participates in many parts of the genetic program mediating T lymphocyte activation and growth. Nuclear expression of NF-κB occurs after its induced dissociation from its cytoplasmic inhibitor IκBα. Phorbol ester and tumor necrosis factor–alpha induction of nuclear NF-κB is associated with both the degradation of preformed IκBα and the activation of IκBα gene expression. Transfection studies indicate that the IκBα gene is specifically induced by the 65-kilodalton transactivating subunit of NF-κB. Association of the newly synthesized IκBα with p65 restores intracellular inhibition of NF-κB DNA binding activity and prolongs the survival of this labile inhibitor. Together, these results show that NF-κB controls the expression of IκBα by means of an inducible autoregulatory pathway.

The NF-κB transcription factor participates in the regulation of multiple cellular genes involved in the immediate early processes of immune, acute phase, and inflammatory responses (*1*). NF-κB has also been implicated in the transcriptional activation of several viruses, most notably the type 1 human immunodeficiency virus (HIV-1) (*2, 3*). In resting $CD4^+$ T lymphocytes, which serve as a primary cellular target for HIV-1, the heterodimeric NF-κB complex is sequestered in the cytoplasm as an inactive precursor complexed with an inhibitory protein, IκB (IκBα) (*4, 5*). Expression of the active nuclear form of NF-κB, composed of 50- and 65-kD subunits, is post-translationally induced (*1, 6*) by various stimuli including mitogens such as phorbol 12-myristate 13-acetate (PMA) (*2, 6–8*) and cytokines including interleukin-1 (IL-1), IL-2, and tumor necrosis factor–alpha (TNF-α) (*9*). These stimulants appear to activate NF-κB by inducing the phosphorylation and release of IκB (*10*), thereby allowing the rapid translocation of NF-κB from the cytoplasm to the nucleus (*4, 7, 10, 11*). However, the biochemical mechanism underlying the transient nature of NF-κB induction remains poorly understood. In the present study, an autoregulatory pathway involving NF-κB induction of its own inhibitor is identified.

To assess the dynamics of IκB protein expression occurring during T lymphocyte activation, subcellular fractions from human Jurkat T cells were treated with various inducing agents and then subjected to immunoblotting (Fig. 1A) and gel retardation (Fig. 1B) analyses. A 37-kD protein was readily detected in cytoplasmic extracts from unstimulated cells with peptide antiserum raised against the COOH-terminus (Fig. 1A) or NH_2-terminus (*12*) of IκBα″ (previously referred to as MAD-3) (*13*), a member of a growing family of proteins known to inhibit NF-κB DNA binding activity in vitro (*13, 14*) and in vivo (*15, 16*). This endogenous immunoreactive protein comigrated with authentic IκBα present in COS-7 cells transfected with an ex-

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