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# Altered Cell Cycle Kinetics, Gene Expression, and $G_1$ Restriction Point Regulation in *Rb*-Deficient Fibroblasts

RAFAEL E. HERRERA,[1] VALERIE P. SAH,[2] BART O. WILLIAMS,[2] TOMI P. MÄKELÄ,[1] ROBERT A. WEINBERG,[1,2] AND TYLER JACKS[2,3]*

*Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142,[1] and Howard Hughes Medical Institute, Center for Cancer Research,[3] and Department of Biology,[2] Massachusetts Institute of Technology, Cambridge Massachusetts 02139*

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**Fibroblasts prepared from retinoblastoma (*Rb*) gene-negative mouse embryos exhibit a shorter $G_1$ phase of the growth cycle and smaller size than wild-type cells. In addition, the mutant cells are no longer inhibited by low levels of cycloheximide at any point in $G_1$ but do remain sensitive to serum withdrawal until late in $G_1$. Certain cell cycle-regulated genes showed no temporal or quantitative differences in expression. In contrast, cyclin E expression in *Rb*-deficient cells is deregulated in two ways. Cyclin E mRNA is generally derepressed in mutant cells and reaches peak levels about 6 h earlier in $G_1$ than in wild-type cells. Moreover, cyclin E protein levels are higher in the $Rb^{-/-}$ cells than would be predicted from the levels of its mRNA. Thus, the selective growth advantage conferred by *Rb* gene deletion during tumorigenesis may be explained in part by changes in the regulation of cyclin E. In addition, the mechanisms defining the restriction point of late $G_1$ may consist of at least two molecular events, one cycloheximide sensitive and pRb dependent and the other serum sensitive and pRb independent.**

The retinoblastoma (*Rb*) tumor suppressor gene is inactivated in a wide range of human tumors. Its encoded protein, pRb, has been implicated in cell cycle regulation and is known to regulate members of the E2F family of transcription factors. These factors, in turn, control the expression of a variety of genes expressed in the $G_1$ and S phases of the cell cycle (34). Therefore, loss of *Rb* function might be expected to result in deregulated gene expression and abnormal cell cycle progression.

pRb has been shown to be phosphorylated at a particular stage of the cell cycle (2, 4, 6, 11). This phosphorylation occurs late in $G_1$ and is responsible for the inactivation of pRb. This event is thought to be critical for passage into S phase. Therefore, a characterization of the mediators of pRb phosphorylation and of the consequences of this event is central to an understanding of the molecular details of cellular progression through $G_1$ and into S.

Recently it has been shown that cyclin-dependent kinases (CDKs), in concert with their regulatory cyclin subunits, can phosphorylate pRb (10, 14, 16). The cyclin/CDKs of particular interest are cyclin E/CDK2 and cyclin D/CDK4,6. These molecules are active in $G_1$, the time of pRb phosphorylation, and indeed have been shown to phosphorylate pRb in vitro and in transfection studies. In addition, these regulatory proteins are essential for transit through $G_1$, and their premature activation leads to an accelerated $G_1$ (28, 31). Thus, cyclin/CDKs are prime candidates for the pRb kinases.

Also occurring late in $G_1$ is the mammalian restriction (R) point (29). The R point was defined by Pardee (29) as the position in $G_1$ beyond which cells are committed to continue into S phase. Three criteria are used to define this point. After the R point, cells (i) no longer need serum to continue into S, (ii) are no longer sensitive to inhibition by transforming growth factor β and (iii) are no longer sensitive to low levels of the protein synthesis inhibitor cycloheximide. Therefore, beyond the R point, cells are no longer responsive to inhibitory signals and no longer need stimulatory signals for progression into S.

The precise roles of pRb in controlling cell proliferation have been difficult to define, since experiments designed to analyze pRb function usually have been conducted with tumor cells that have suffered a number of other mutations in addition to alteration of *Rb*. These other changes complicate analysis of the role of pRb itself. To circumvent this problem, we have analyzed fibroblasts prepared from *Rb*-deficient mouse embryos; these cells are genetically matched with those prepared from wild-type embryos with the exception of the known mutation in *Rb* itself (15).

## MATERIALS AND METHODS

**Cell cycle analyses.** Early-passage (<8) primary fibroblasts isolated from *Rb* mutant and wild-type embryos at 12.5 days of gestation (15) were used in all experiments. Experiments were carried out by comparing cells at identical passage numbers obtained from littermate embryos. Cells were labeled with bromodeoxyuridine-propidium iodide and analyzed on a Becton Dickinson FACScan for cell cycle or size analysis. For [$^3$H]thymidine incorporation experiments, cells were starved in serum-free Dulbecco modified Eagle medium for >48 h before being restimulated with 15% serum for the indicated times. [$^3$H]thymidine labeling was for 30 min before lysis and counting.

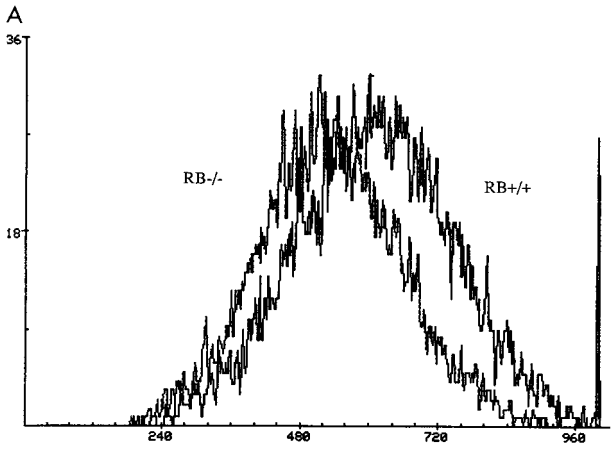
**Immunoblot analyses and kinase assays.** Immunoblot analyses were performed by standard procedures (1). Antibodies to cyclin proteins were from rabbit polyclonal serum. Specificity of the antibodies was confirmed by detection of recombinant cyclin proteins and migration in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Kinase assays were performed as described previously (22), using equal amounts of protein for each immunoprecipitation. Antibodies to CDK2 were from Santa Cruz Biotechnology. Quantification of the kinase assay was by densitometric scanning.

**RNA analyses.** RNA purification and Northern (RNA) analyses were performed by standard procedures (1). The probes were derived from cDNAs of the respective genes. Quantification was by densitometric scanning.

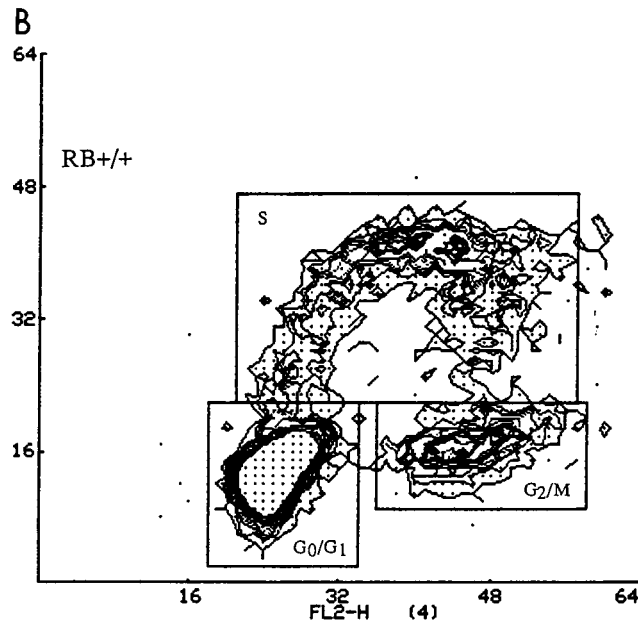
## RESULTS AND DISCUSSION

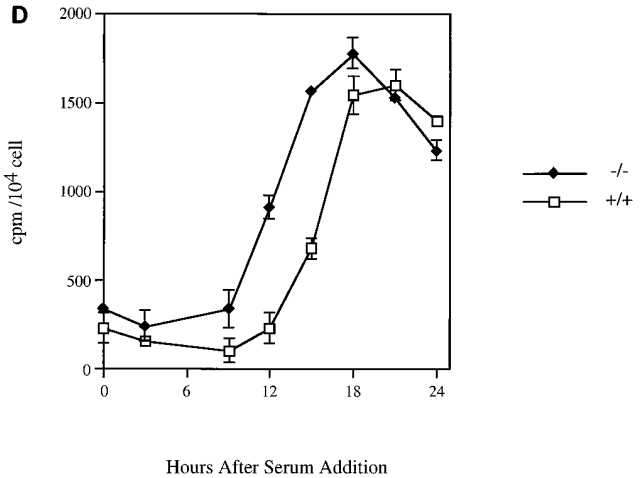
Microscopic examination and flow cytometric analysis revealed that the diameter of the $Rb^{-/-}$ cells was approximately 85% of that of the wild-type cells (Fig. 1A). The calculated 40% smaller volume of the $Rb^{-/-}$ cells suggested a shorter

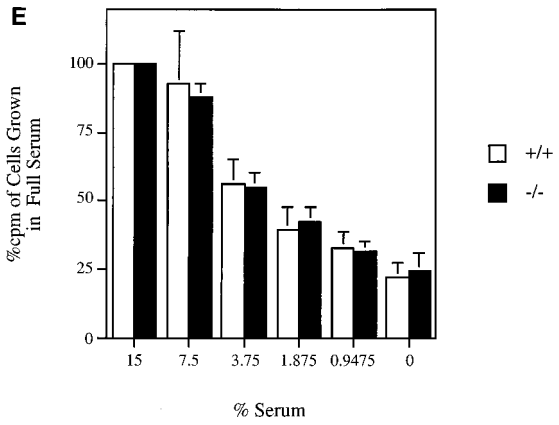
* Corresponding author. Phone: (617) 253-0262. Fax: (617) 253-9863.




| C | % $G_0/G_1$ | % S | % $G_2$/M |
|---|---|---|---|
| RB+/+ Cells | 65.5 +/- 6.9 | 18.5 +/- 7.1 | 15.6 +/- 4.9 |
| RB-/- Cells | 45.7 +/- 7.8 | 31.2 +/- 7.8 | 22.1 +/- 9.4 |



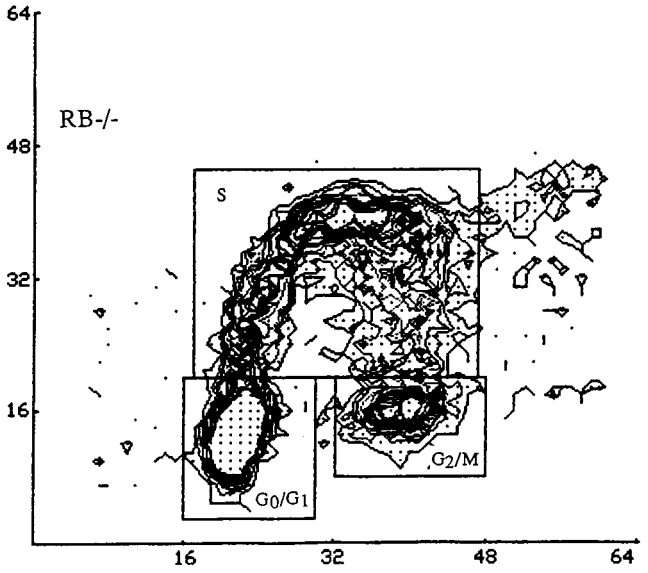




FIG. 1. Altered size and cell cycle kinetics in $Rb^{-/-}$ fibroblasts. (A) Forward versus side-scatter flow cytometric analysis. Size is plotted on the *x* axis, and cell number is plotted on the *y* axis. Curves from mutant and wild-type cells are indicated. (B) FACScan analysis of asynchronously growing mutant and wild-type cells labeled with bromodeoxyuridine-propidium iodide to determine the percentage of cells in each cell cycle phase. The $G_0/G_1$, $G_2$/M, and S phases are labeled. (C) Summary of FACScan analyses, with standard deviations, from a total of 14 and 16 experiments from $Rb^{+/+}$ and $Rb^{-/-}$ cells, respectively. The observed compensatory increase in S seen in the mutant cells has also been found in fibroblasts overexpressing cyclin E (28). (D) [$^3$H]thymidine incorporation of mutant and wild-type cells labeled at different times after release into $G_1$ by readdition of serum to starved cells. Data are derived from an experiment performed in triplicate; standard deviations are indicated by error bars. Similar results were obtained from cells derived from at least 10 different $Rb^{+/+}$ and $Rb^{-/-}$ embryos. (E) Thymidine incorporation of mutant and wild-type cells labeled after growth in reduced concentrations of serum. Cells were grown for 3 days at the serum concentrations indicated and then labeled with [$^3$H]thymidine. The *y* axis is expressed as the percent incorporation of cells grown in 15% serum. Data shown were derived from an experiment performed in triplicate; standard deviations are indicated by error bars.

growth cycle. Therefore, we determined the length of the cell cycle phases by flow cytometry of asynchronously growing cells. As shown in Fig. 1B and C, while the percentages of $Rb^{-/-}$ and wild-type cells in $G_2$ were comparable, there was a 20% reduction in the number of mutant cells in $G_1$ and a concomitant increase of 12% of those in S. Since the percentage of cells in each cell cycle phase reflects the time required for each cell type to transit that phase, we concluded that pRb influences the timing of $G_1$ and that its absence leads to an abbreviated $G_1$ and reduced cell size. However, because of a lengthened S phase, the overall doubling time of the pRb-deficient cells was comparable to that of their wild-type counterparts (not shown).

To confirm these altered cell cycle kinetics, mutant and wild-type cells were starved of serum and then released from $G_0$ by the readdition of serum. S-phase entry was monitored by measuring incorporation of [$^3$H]thymidine into DNA. Mutant cells synchronized in this fashion exhibited a $G_1$ phase that is 3 to 4 h shorter than that of their wild-type counterparts (Fig. 1D), reinforcing the results from flow cytometry. Together, these observations provide direct evidence indicating that pRb indeed is an important regulator of cell cycle progression.

It was of interest to determine if the mitogenic effects of serum worked in part through inactivation of pRb. Therefore, we determined if the $Rb^{-/-}$ cells had obtained some level of serum independence for growth. $Rb^{-/-}$ and $Rb^{+/+}$ cells were grown in various concentrations of serum for 3 days before being pulse-labeled with [$^3$H]thymidine. As shown in Fig. 1E, the two cell types exhibit very similar degrees of labeling at all concentrations of serum used. This result suggests that while serum stimulation of growth may work in part by inactivation of pRb, other molecular events are required for cells to proceed through the cell cycle.

As described above, the critical checkpoint of $G_1$ has been termed the R point by Pardee (29). The R-point transition and the onset of pRb phosphorylation and inactivation occur at similar times in mid/late $G_1$, suggesting a connection between these two processes. Therefore, it was of interest to determine whether R-point control was maintained in the $Rb^{-/-}$ cells. Mutant and wild-type cells were growth arrested by serum starvation and then allowed to reenter the cell cycle by readdition of serum. At various points thereafter, either serum was removed (Fig. 2A) or cycloheximide was added to the cells to a final concentration of 50 ng/ml (Fig. 2B). The R point, as defined by escape from serum dependence, was found to occur at similar times relative to the onset of S phase in both cell types (Fig. 2A). Because the $Rb^{-/-}$ cells progress through $G_1$ more rapidly than wild-type cells and enter S phase 3 to 4 h earlier following serum addition (Fig. 1D), the R point is similarly shifted by this amount of time relative to controls (Fig. 2A). Importantly, the $Rb^{-/-}$ cells remain dependent on serum factors up to the R-point transition (Fig. 1E). In striking contrast, the cycloheximide R point appeared to be largely if not completely absent in the $Rb^{-/-}$ cells (Fig. 2B); thus, $Rb^{-/-}$ cells escaped cycloheximide inhibition rapidly after serum restimulation. These results indicated that the R-point transition depends on at least two distinct, separable molecular processes, one cycloheximide sensitive and pRb dependent and the other serum sensitive and pRb independent.

A shorter $G_1$ and smaller size have also been observed in cells that overexpress several of the $G_1$ cyclin genes (28, 31–33). For this reason, we determined the levels of cyclin proteins at various points of the cell cycle in the $Rb^{-/-}$ and wild-type cells by immunoblotting. The expression of $G_1$ cyclins was variably affected by the absence of pRb (Fig. 3A). Cyclin D1 levels were slightly elevated (approximately two- to fourfold) in

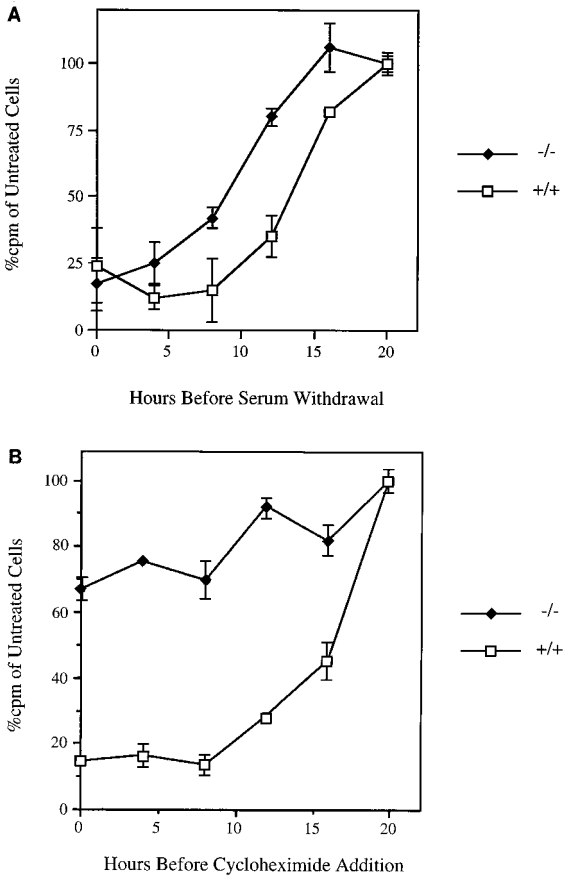



FIG. 2. Analysis of the R point of late $G_1$ in $Rb^{-/-}$ and $Rb^{+/+}$ primary fibroblasts. (A) Mutant and wild-type cells were growth arrested by serum starvation and then allowed to reenter the cell cycle by readdition of serum. At various points thereafter, serum was removed. Entry into S was measured by [$^3$H]thymidine incorporation at 20 h after serum restimulation. (B) As described for panel A except that instead of serum removal, cycloheximide was added to the cells to a final concentration of 50 ng/ml. The *y* axis is expressed as the percent counts per minute of samples serum stimulated for 20 h without subsequent serum withdrawal or cycloheximide addition. Results from representative experiments are shown. Data shown were derived from an experiment performed in triplicate; standard deviations are indicated by error bars. Similar results were obtained from cells derived from at least 10 independent $Rb^{+/+}$ and $Rb^{-/-}$ embryos.

the $Rb^{-/-}$ cells at different points in $G_1$. In contrast, cyclin E protein levels were vastly increased in the pRb-deficient cells regardless of their position in $G_0$ or $G_1$. Quantitation of autoradiograms indicated that the mutant cells contain approximately 10 times more cyclin E protein (data not shown). In addition, the slight induction of cyclin E protein levels occurring in wild-type cells late in $G_1$ was observed earlier in the $Rb^{-/-}$ cells (Fig. 3A; compare 18- and 24-h time points in wild-type cells with 6- and 12-h time points in mutant cells). Cyclin A protein levels were slightly elevated in the mutants early in $G_1$ as well as in $G_0$. These observed increases could be a consequence of the premature and highly elevated expression of cyclin E in the pRb-deficient fibroblasts or a direct regulation of cyclin A expression by pRb.

We next addressed whether the increased levels of the cyclin E and A proteins present in the mutant cells also led to a corresponding increase in CDK2 kinase activity. Accordingly, CDK2 complexes were immunoprecipitated from wild-type and pRb-deficient cells and used to phosphorylate histone H1

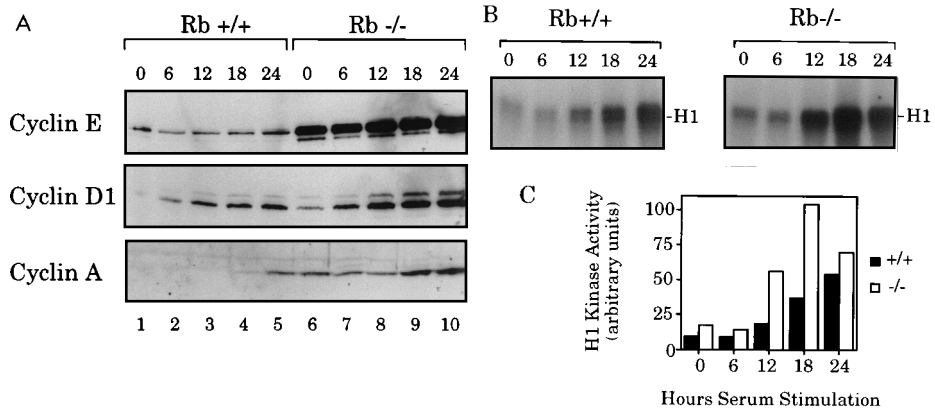



FIG. 3. (A) Immunoblot analysis of protein lysates prepared after the readdition of serum to starved cells. Blots contain total protein lysates from cells stimulated with serum for the indicated times. Cyclin proteins were detected by polyclonal rabbit antibodies. Protein amounts were normalized by spectrophotometric methods and Ponceau S staining of the nitrocellulose filters. (B) Histone H1 kinase activity of anti-CDK2 immunoprecipitates prepared after the readdition of serum to starved cells for the indicated times. (C) Quantification of the anti-CDK2 immunoprecipitable histone H1 kinase activity.

in vitro. Figures 3B and C show that immunoprecipitates from the $Rb^{-/-}$ cells display higher H1 kinase activities throughout $G_1$. In addition, the peak of kinase activity occurs earlier in the mutant cells, and this difference is comparable to their shortening of $G_1$. It should be noted that the kinase activities were measured by using CDK2 antibodies in the immunoprecipitations. Therefore, both cyclin A and cyclin E could have contributed to increased CDK2 activity. In addition, as in wild-type cells, $Rb^{-/-}$ cells undergo a serum-dependent activation of CDK2 function, despite constitutive high levels of cyclin E protein. Indeed, this pRb-independent kinase activation may account for the observed serum dependence of the $Rb^{-/-}$ cells (Fig. 1E and 2A).

We next performed Northern blot analysis of RNA prepared at various times after the readdition of serum to determine if the dramatic increase in cyclin E protein levels in the $Rb^{-/-}$ cells was due to differences in levels of cyclin E mRNA. Expression of the cyclin E gene has been shown to be normally induced in late $G_1$ several hours before entrance into S phase (8, 11, 20, 21). As shown in Fig. 4A and C, peak levels of cyclin E mRNA expression were seen 6 h earlier in the mutant cells. This difference is comparable to the overall shortening of $G_1$ seen in the $Rb^{-/-}$ cells. In addition, cyclin E mRNA levels were elevated in all parts of $G_1$ in the mutant cells. However, as in the CDK2 kinase activity profile (Fig. 3), an induction of cyclin E mRNA occurs in the $Rb^{-/-}$ cells, suggesting that the cyclin E gene is under complex transcriptional control, only a part of which is responsive to pRb inactivation.

Work of others has demonstrated that the ectopic expression of cyclin E causes a substantially shortened $G_1$ phase of the cell cycle, smaller cells, and a longer S phase (28, 32, 33). These observations suggest that the entrance into the late phase of $G_1$ is controlled by the availability of cyclin E mRNA and protein and can be advanced by prematurely elevated protein levels. The pRb-deficient cells show a phenotype strikingly similar to that of these cyclin E-overproducing cells, including elevated cyclin E protein levels at all points of the cell cycle. It is interesting that elevated levels of cyclin E have recently been observed in several human tumors (17, 18).

Transcriptional up-regulation of the *TK* and cyclin A genes, which normally follows cyclin E induction, was also accelerated in the $Rb^{-/-}$ cells (Fig. 4A and data not shown). Whether this is a direct result of the absence of *Rb* function or an indirect effect of the overexpression of cyclin E is unclear.

In contrast to the changes in gene expression observed in mid/late $G_1$, transcription of the immediate-early gene c-*fos* and transcription of two delayed-early genes, c-*myc* and the cyclin D1 gene, showed identical patterns (Fig. 4A and B). This finding suggests that pRb does not exert any direct control on gene expression early in $G_1$ and thus contrasts with results of others implying pRb-mediated control of c-*fos* and the cyclin D1 gene and E2F-mediated control of c-*myc* (13, 19, 24, 35, 36). Therefore, the influence of pRb on the timetable of $G_1$ progression appears to be limited largely to its effects on the timing of the induction of cyclin E mRNA and subsequently occurring processes. This suggests, in turn, that the $G_1$ phase of the cell cycle is divided into two periods demarcated by the mid/late $G_1$ event of cyclin E mRNA induction.

As described above, the peak levels of cyclin E mRNA are about twofold higher in the *Rb*-deficient cells but the levels of protein are about 10 times higher. This disparity suggests that pRb may also influence posttranscriptional control mechanisms governing cyclin E expression. The nature of these mechanisms and their regulation by pRb are unknown at this time.

The detailed mechanisms underlying the negative regulation of cyclin E expression by pRb are not revealed by the present work. One possibility is suggested by the observation (10a) that the cyclin E gene promoter contains a number of E2F sites, the activity of which may be inhibited by pRb. Independent of mechanism, the observed ability of pRb to antagonize cyclin E expression would seem to stand in opposition to earlier studies demonstrating that cyclin E operates to antagonize pRb function (14). In this earlier work, ectopic expression of cyclin E was found to cause the hyperphosphorylation of pRb and its functional inactivation. The two sets of observations can be reconciled by the existence of a self-reinforcing, positive feedback loop that is established in late $G_1$, in which cyclin E participates in the functional inactivation of pRb while the resulting inactivated pRb permits the ongoing expression of cyclin E. We note that a positive feedback loop has also been proposed to describe the control of late $G_1$ transit in the yeast *Saccharomyces cerevisiae* and fruit flies (5, 7, 9, 25–27).

Unexplained by the present work is the disparity between the reduced $G_1$ phase of the pRb-deficient fibroblasts in culture and the relatively normal development of $Rb^{-/-}$ embryos up until day 13 of gestation (15), particularly in light of the presumed importance of $G_1$ in various growth control and

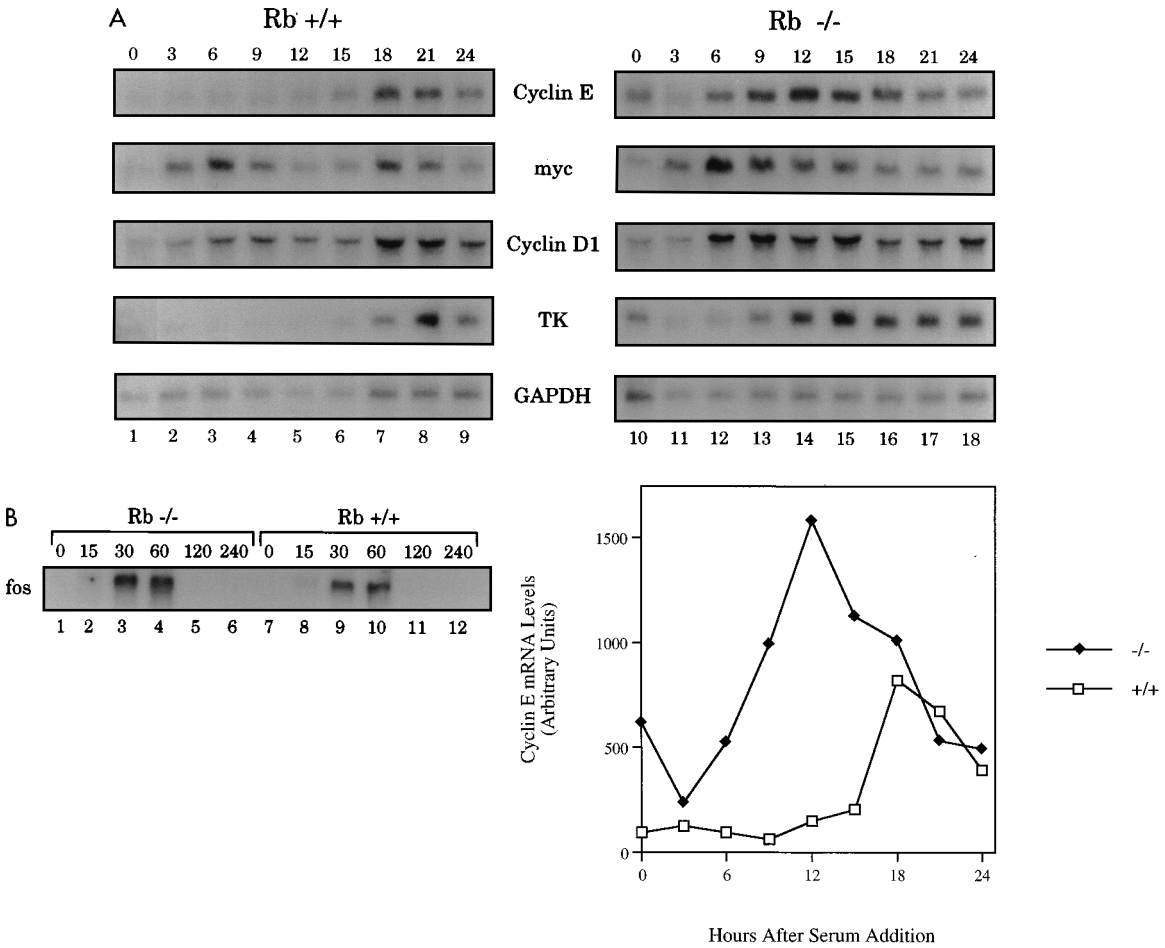



FIG. 4. Northern blot analyses of RNA prepared after the readdition of serum to starved cells. (A) Blots containing poly(A)$^+$ RNA purified from cells stimulated with serum for the indicated times (hours) were hybridized to the indicated radiolabeled cDNA probes. Samples of RNA from $Rb^{+/+}$ and $Rb^{-/-}$ cells are indicated. As shown by the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) hybridization, slightly more RNA was loaded in the wild-type 18-, 21-, and 24-h samples. (B) A blot containing total RNA purified from cells stimulated with serum for the indicated times (minutes) was hybridized to a radiolabeled c-*fos* probe corresponding to the third exon of the human gene. Equal loading was ensured by UV shadowing of the membrane before hybridization (12). (C) Quantification of the cyclin E Northern blot in panel A.

differentiation mechanisms. In addition, the relatively normal transcriptional induction of the cell cycle-regulated genes c-*fos*, c-*myc*, and the cyclin D gene observed in pRb-deficient fibroblasts suggests that these genes may be regulated by other non-pRb proteins.

Evidence from a variety of sources indicates that the R point represents a cell cycle landmark that is of central importance in cell growth control (29). Indeed, it has been shown that transformed cells are more resistant to cycloheximide than are their nontransformed counterparts (3, 23, 30). Yet other results indicate that the pRb control mechanisms are disrupted by a variety of ways in many and possibly all types of tumor cells (37). By at least one operational definition—cycloheximide inhibition—R-point control appears to be absent in $Rb^{-/-}$ mouse embryo fibroblasts. This observation, together with the observed contemporaneity of pRb phosphorylation and the R-point transition, suggests that pRb may mediate control of this important growth-regulating checkpoint.

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