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Advances in Brief

Cyclin E and Cyclin A as Candidates for the Restriction Point Protein[1]

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Abstract

Progression of cells into S phase is proposed to be determined by accumulation of a labile protein (the restriction point protein R; A. B. Pardee, Proc. Natl. Acad. Sci. USA, *71:* 1286–1290, 1974). We report here that cyclin E and cyclin A proteins as well as their dependent histone H1 kinases satisfy all of the criteria for the R protein, which includes late G_1 phase increase, an excess delay of appearance after inhibition of protein synthesis in nontransformed cells, and a faster recovery in transformed cells. We suggest that the molecular basis of the R protein could be cyclin production and inactivation.

Introduction

Kinetic experiments from cell biology suggest that a controlling protein must accumulate by the restriction point, which is located in late G_1 phase, before a cell can enter S phase (1–4). A key protein for regulating proliferation, called the R protein,[3] was proposed by this laboratory to have the following three properties: (*a*) it is synthesized in G_1; (*b*) it is unstable, with a half-life of 2.5 hours, in nontransformed cells; and (*c*) it is stabilized or overproduced in tumor cells (1–4). More recently, genetic and biochemical approaches together have indicated that protein kinases of about M_r 34,000 (encoded by the *cdc2* family of genes, *e.g., cdc2, cdk2*) are activated through interactions with the products of another family of genes, the cyclins (5–7). With the discovery of G_1 cyclins in both yeast and mammalian cells it was proposed that the R protein might be a mammalian G_1 cyclin(s) (6, 7). However, this has not yet been shown in experiments such as were used originally to propose the existence of the R protein (2). In the study presented here, by performing CHX pulse-chase experiments in both nontransformed A31 and BPA31 cells, we found that cyclin E and cyclin A as well as their dependent kinase activities satisfy all the criteria for the R protein.

Materials and Methods

Materials. [γ-^{32}P]ATP (3000 Ci/mmol) and [^{3}H]thymidine (70–90 Ci/mmol) were from NEN Research Products. Sodium vanadate (S454-50) was from Fisher. Histone H1 (Type III-S, H 5505), cAMP-dependent kinase inhibitor peptide (P 8140), aprotinin (A 1153), leupeptin (L 2884), soybean trypsin inhibitor (T 9003), benzamidine (B 6506), NaF (S 6521), β-glycerophosphate (G 6251), cycloheximide (C 6255), and all other chemicals were from Sigma. p13^{suc1} agarose (PF001A) and protein G PLUS/protein A-agarose (IP05) were from Oncogene Science. Purified polyclonal antibody raised against the carboxyl terminus of human p34^{cdc2} (3398SA) was purchased from Gibco BRL. Two purified antibodies against the carboxyl terminus of *cdk2* were used in both immunoprecipitation and Western blot assays: one of them was purchased from UBI (06-148) and the other was a kind gift from Drs. N. Dyson and E. Harlow (Massachusetts General Hospital Cancer Center, Charlestown, MA). Affinity-purified rabbit polyclonal IgG against recombinant human cyclin A (PC30) and cyclin B1 (PC29) were from Oncogene Science; antisera to human cyclin A and cyclin B were a generous gift from Dr. J. Pines (Wellcome/CRC Institute, Cambridge, England) (8). Two polyclonal antisera to human cyclin E were used. The anti-cyclin E antiserum used in the immunoprecipitation assay was kindly provided by Drs. A. Koff and J. M. Roberts (Fred Hutchinson Cancer Research Center, Seattle, WA) (9), and another anti-cyclin E used in Western blot analysis was from Dr. S. I. Reed (Scripps Research Institute, La Jolla, CA) (10).

Cell Culture, Cell Synchronization, and Nuclear Labeling. A31 and BPA31 cells were grown as monolayer cultures, and cell synchronization after serum deprivation was achieved as described (3). Briefly, after growth in 10% calf serum to about 30–50% confluence, A31 cells were shifted to 0.4% calf serum for ~60 h while BPA31 cells were shifted to 0.2% calf serum for ~84 h. After growth arrest, serum levels were increased to 10%. Under these conditions, A31 and BPA31 cells enter into S phase with similar kinetics, monitored by the incorporation of [^{3}H]thymidine (continuous labeling). In the studies using CHX, the inhibitor (1.0 µg/ml) was added to cells 12 h after serum stimulation. After 5 h of treatment, cells were washed once with, and then refed with, medium containing 10% serum.

Preparations of Nuclear Extracts, p13^{suc1}-Agarose Pellets, and Immunoprecipitates. Nuclear extracts of proteins were prepared from cells essentially according to the published method (11). Treatment of a nuclear extract with p13^{suc1}-agarose beads was performed as described (12). The nuclear extract was incubated for 2 h under constant vortexing at 4°C with p13^{suc1}-agarose (5 µg protein/5 µl p13^{suc1} beads/reaction) in 25 µl of buffer B (50 mM Tris, pH 7.4; 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40) with a freshly added cocktail of protease inhibitors (10 µg/ml of aprotinin, leupeptin, and soybean trypsin inhibitor and 100 µM benzamidine) and phosphatase inhibitors (1 mM sodium vanadate, 2 mM EGTA, 5 mM NaF, 12 mM β-glycero-phosphate, and 1 mM ATP). After 10 min of centrifugation at 1000 rpm, the p13^{suc1}-agarose pellets were washed twice with 1 ml of buffer B and three times with 1 ml of buffer K (50 mM Tris, pH 8.0; 10 mM $MgCl_2$; and 1 mM dithiothreitol) containing both protease and phosphatase inhibitors and immediately used for the H1 kinase assay. For the immunoprecipitation of nuclear proteins (13), a specific antibody (the amount is indicated in the figure legends) was preincubated with a nuclear extract (5 µg) for 2 h, followed by adding 10 µl of protein G-PLUS:protein A-agarose (1:1 suspension) in buffer B (total volume, 25 µl). After 2 h of vortexing of this mixture, the pellet was washed with buffer B and buffer K and used for H1 kinase assay.

Histone H1 Kinase and Western Blot Assays. The H1 kinase assay was performed as described (12–14). The ECL Western blot assay was performed according to Amersham instructions. Briefly, nuclear protein samples were electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel and then electrophoretically transferred to a nitrocellulose membrane (14). The nitrocellulose blot was blocked, washed, and incubated with the first antibody (for dilution conditions, see figure legends). After washing, the filter was then incubated with a second antibody that was conjugated with horseradish peroxidase. Finally, the filter was incubated with the detection reagents (RPN 2109; Amersham) and exposed to a Hyperfilm-ECL (RPN 2103). For reprobing, the filter was incubated in a stripping buffer (100 mM 2-mercaptoethanol;

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[3] The abbreviations used are: A31, BALB/c 3T3 clone A31 cells; BPA31, benzo[α]-pyrene-transformed A31 cells; p34^{cdc2}, mouse homologue of M_r 34,000 protein encoded by the *Schizosaccharomyces pombe cdc2* gene or the *Saccharomyces cerevisiae CDC28* gene; cdk, cyclin-dependent kinase; CHX, cycloheximide; H1K, histone H1 kinase; R protein, the restriction point protein.

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2% sodium dodecyl sulfate; 62.5 mM Tris, pH 6.7) at 50°C for 30 min, washed, and then immunodetected as described above.

Results and Discussion

We investigated the possibility of whether a G_1/S cyclin protein(s) possess all the properties of the R protein in the following three tests.

Test 1: Activation in Late G1 in Nontransformed Cells. We first performed an experiment to measure the increases of H1 kinases in late G_1 associated with cyclin or *cdk* proteins (designated as Cyc-H1K or *cdk*-H1K, respectively), the first criterion for the R protein (4). We used antibodies against human cyclins (A, B, E) and *cdks (cdc2, cdk2)*, as well as $p13^{suc1}$ beads which bind to several *cdk*/H1 kinases including *cdk2* (15), to precipitate the corresponding proteins and then measured their H1 kinase activities. All of these H1K activities were low in G_0 cells, but they increased at different phases of the cell cycle (Fig. 1*a*). CycE-H1K increased before 12 h and peaked at 17 h, at which time about 40% of nuclei were labeled by $[^3H]$thymidine (measured by continuous labeling) (Fig. 1*b*). CycA-H1K gradually increased after 12 h and reached its highest value at 36 h, parallel to the kinetics of DNA synthesis as measured by nuclear labeling. In contrast, CycB-H1K did not increase until 36 h (Fig. 1*b*). The *cdk2*-H1K increased at 12 h and dropped after S phase (36 h; Fig. 1*c*), supporting its interaction with both cyclin E and cyclin A (reviewed in Refs. 6, 9, and 10). The $p34^{cdc2}$-H1K showed a pattern similar to that of CycA-H1K, suggesting that it mainly interacts with cyclin A before 36 h. The p13-H1K had two major increases, one before 17 h, which may be derived from cyclin E, while another increase took place before 36 h, which is probably from both cyclin A and cyclin B (Fig. 1*b*). Similar patterns of cyclin E/*cdk2* and cyclin A/*cdk2* kinases were discovered in other systems (6, 9, 10). Our results support prior data (6, 9, 10, 16) that cyclin E/*cdk2* and cyclin A/*cdk2* are late G_1- and S-phase-specific kinases that are involved in the entry of cells into S.

We then performed Western blot analyses to determine whether cyclin E and cyclin A proteins accumulate in G_1/S. An antiserum to human cyclin E recognized mainly two bands with approximate molecular masses of 52 (p52) and 65 kilodaltons (p65) (Fig. 2*a*). p52 probably represents the mouse cyclin E protein since accumulation of this protein correlates well with CycE-H1K during the cell cycle: very low in G_0 cell nuclear extract, increased at G_1/S (around 12 h), peaked at 18 h (about 8-fold higher than at 0 h), and decreased at 22 h (compare Figs. 1 and 2). In contrast, p65 was relatively constitutively expressed during the cell cycle, and its nature remains unknown. A mouse protein of p55, identified by an antiserum to human cyclin A (Fig. 2*a*), was not present in G_0 cells but increased after cells enter S phase (about 38-fold higher at 22 h than at 0 h), coincident with CycA-H1K (compare to Fig. 1). When anti-*cdk2* was used, a major band of p38 with two minor bands of p30 and p41 were detected; all of them were present in G_0 nuclear extracts and increased by only 2-fold after cells entered S phase (Fig. 2*b*; compare 22 h and 0 h). Similar isoforms of *cdk2* protein had been found in other systems (9, 10). These data strongly suggest that accumulation of cyclin E and cyclin A proteins at G_1/S regulate the induction of their *cdk* kinase activities.

Test 2: An Excess Delay of Appearance after the Inhibition of Protein Synthesis in Nontransformed Cells. We expected that CycE-H1K and CycA-H1K are labile in nontransformed A31 cells, the second criterion for the R protein (4). Indeed, after treatment with CHX, we observed 2–4-fold decreases in these H1K kinases (Fig. 3, *Lanes 1–3*). About 4 hours after CHX was removed, H1K activities started to recover (note displacement of the curves *versus* Fig. 1); further chasing revealed large increases in these activities (Fig. 3, A31 cells).

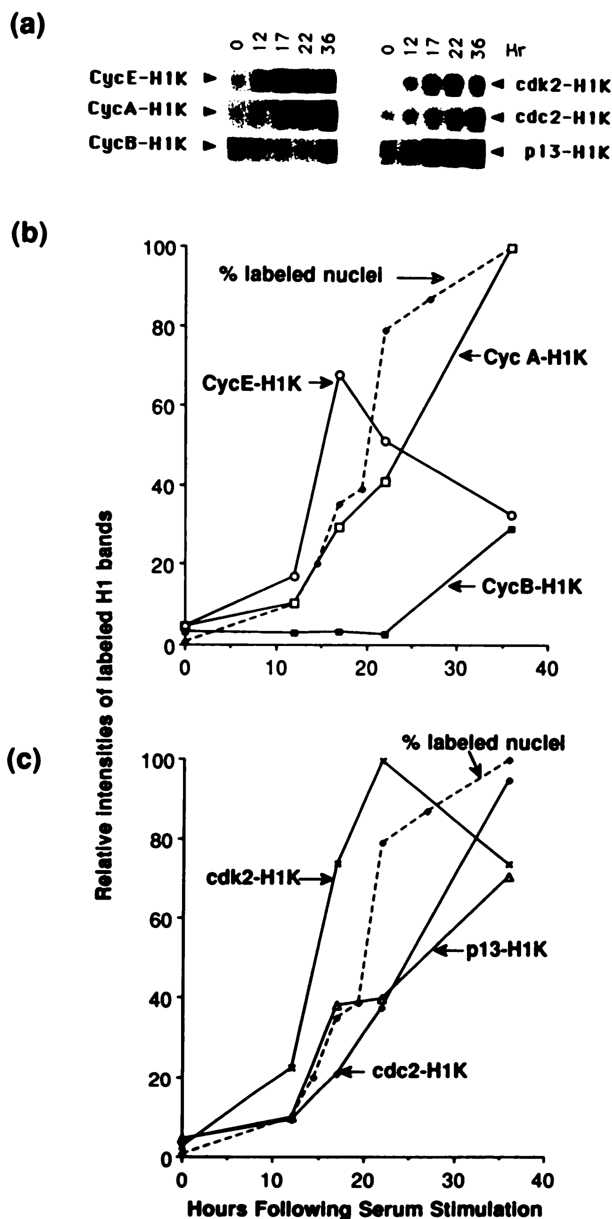

Fig. 1. Cell cycle patterns of H1 kinases associated with different cyclin, *cdk* proteins, or $p13^{suc1}$ beads. A31 cells were made quiescent by serum starvation, restimulated with 10% serum, and harvested at the times indicated at the *top*. Percentage of labeled nuclei (*dashed lines* in *b* and *c*), measured by continuous labeling with $[^3H]$thymidine incorporation, demonstrated that cells began entering S phase after 12 h; by 17 h about 40% and by 22 h about 80% of the cells had entered S phase. The histone H1 kinase assays using immunoprecipitates of nuclear extracts prepared at each time point were performed as described in "Materials and Methods." In each reaction, 5 µg of crude nuclear extract were immunoprecipitated by a specific antiserum against a human cyclin (E, A, and B, 1 µl/reaction) or by a purified antibody to the COOH terminus of *cdk2* or *cdc2* (1 µl/reaction), or $p13^{suc1}$ beads (5 µl/reaction), as indicated in *a*. Exposure times of autoradiographs were: cyclin E, 3 h; cyclin A, 1 h; cyclin B, 2 h; *cdk2*, 2.5 h; *cdc2*, 3 h; p13, 3 h. The labeled histone H1 bands were scanned, and relative intensities were plotted *versus* hours after serum stimulation. *b*, plot of CycE-H1K, CycA-H1K, and CycB-H1K; *c*, plot of *cdk2*-H1K, *cdc2*-H1K, and p13-H1K. These data are presented to indicate patterns of each kinase activity rather than absolute levels of kinase activities. Comparisons are valid only within the same kinase activity and not between kinase activities.

The lags in both CycE-H1K and CycA-H1K activities caused by CHX pulse treatment may reflect the pulse inhibition of the synthesis of unstable cyclin proteins. Indeed, even though the level of p52/cyclin E was very low before and during the pulse inhibition, it increased 2-3-fold after chasing (Fig. 4*a*). p65 was relatively unchanged during the process. p55/cyclin A protein was almost undetectable at 12 h and during the protein inhibition and gradually in-

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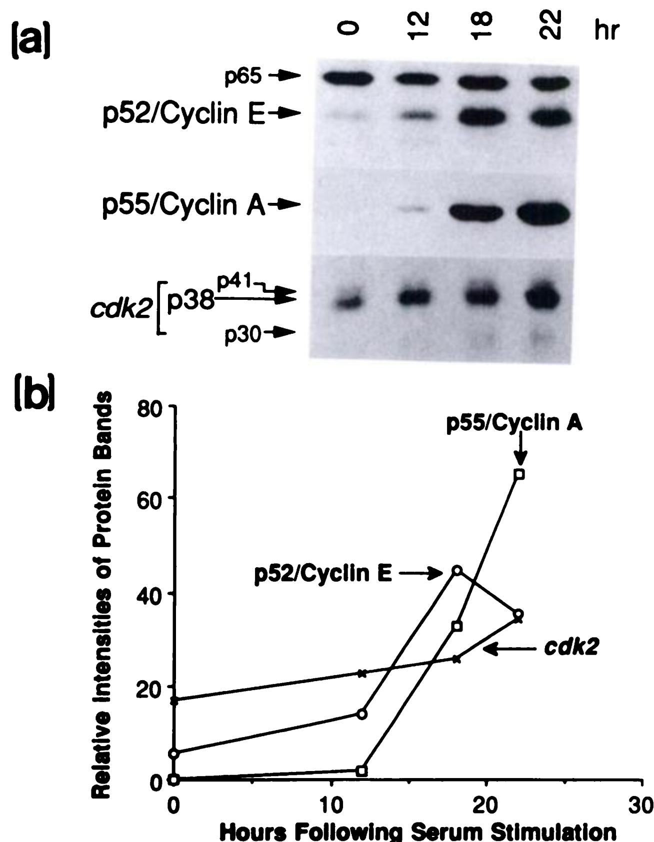

Fig. 2. Cell cycle patterns of cyclin E, cyclin A, and *cdk2* proteins. Nuclear extracts (40 μg/lane), prepared from synchronized A31 cells harvested at the indicated times in hours (*above lanes*) after serum stimulation, were electrophoresed and assayed by Western blot analysis. In *a*, anti-cyclin E at 1:500 and the second antibody at 1:2000 were used; *arrows*, p52/cyclin E and p65. Anti-cyclin A at 1:300 and the second antibody at 1:4000 were used; *arrow*, p55/cyclin A. Anti-*cdk2* at 1:500 and the second antibody at 1:1000 were used; *arrows*, p30, p38, and p41 of *cdk2*. *b*, plot of p52/cyclin E, p55/cyclin A, and p38 (p41)/*cdk2*.

creased about 5 hours after removing CHX (Fig. 4*b*). In contrast, *cdk2* was relatively constitutive during this process (data not shown). Therefore, cyclin proteins are probably the labile component responsible for the regulation of *cdk* kinases.

Test 3: Faster Recovery from CHX Inhibition in Transformed Cells. We expected that transformed BPA31 cells would show no or less delay in H1K appearance at high levels after the pulse of CHX (the third criterion for the R protein) (4). CHX inhibits protein synthesis in A31 and BPA31 cells equally (3). Comparison of cyclin E- and cyclin A-associated H1Ks between BPA31 and A31 cells (Fig. 3) gave results similar to, but more complicated than, those observed from earlier cell biology experiments (3).

First, the levels of CycE-H1K (Fig. 3*a*) and CycA-H1K (Fig. 3*b*) at 12 h were 2–3-fold higher in BPA31 than in A31 cells, as found in multiple experiments. Second, even though CHX reduced these H1Ks in both A31 and BPA31 cells, the remaining activities after the treatment were higher in BPA31 than in A31 cells. Third, both CycE-H1K and CycA-H1K recovered to high levels much earlier in BPA31 cells than in A31 cells.

To determine whether there are such differences in cyclin proteins between BPA31 and A31 cells, we performed Western blot analysis using nuclear extracts from the same preparations. Basically, we observed similar differences in cyclin proteins (Fig. 4) and *cdk* kinases (Fig. 3). At 12 h, cyclin E protein was much higher in BPA31 cells than in A31 cells (Fig. 4*a*), while cyclin A was slightly higher in BPA31 cells (Fig. 4*b*). Second, CHX treatment reduced the accumu-

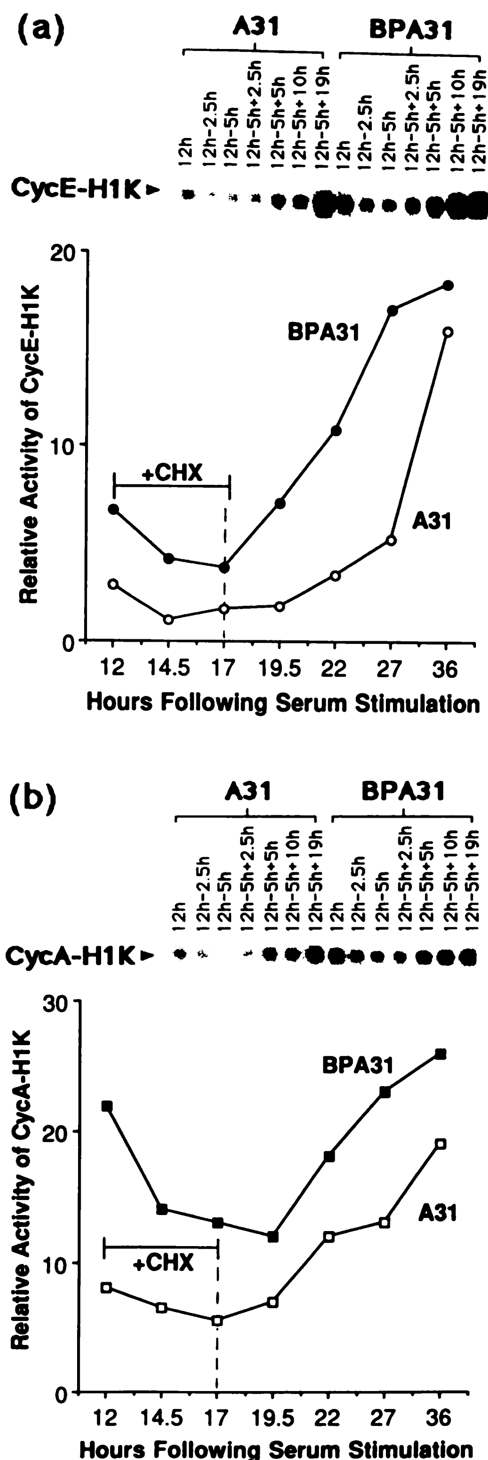

Fig. 3. CHX delays the appearance of H1K associated with cyclin E (*a*) and cyclin A (*b*). A31 and BPA31 cells were released after serum starvation, and 12 h (*12h*) afterward CHX (1 μg/ml) was added for either 2.5 h (*12h–2.5h*) or 5 h (*12h–5h*). After CHX was removed from the 5-h-treated cultures, the cells were grown with fresh 10% serum for 2.5, 5, 10, or 19 h, as indicated at the *top*. Nuclear extracts were prepared, and histone H1 kinase assays were performed with immunoprecipitates (see "Materials and Methods"), using anti-cyclin E (1 μl/reaction) and anti-cyclin A (20 μl/reaction of purified polyclonal antibody against recombinant cyclin A; Oncogene Science). Exposure times of autoradiographs were: cyclin E, 1 h; cyclin A, 8 h. The relative intensities of labeled H1 bands were determined by densitometric tracing and plotted *versus* h during the pulse chase process.

lation of both cyclin E and cyclin A proteins in BPA31 cells (it was difficult to measure decreases of these proteins in A31 cells because of their low basal levels), but the remaining cyclin proteins were

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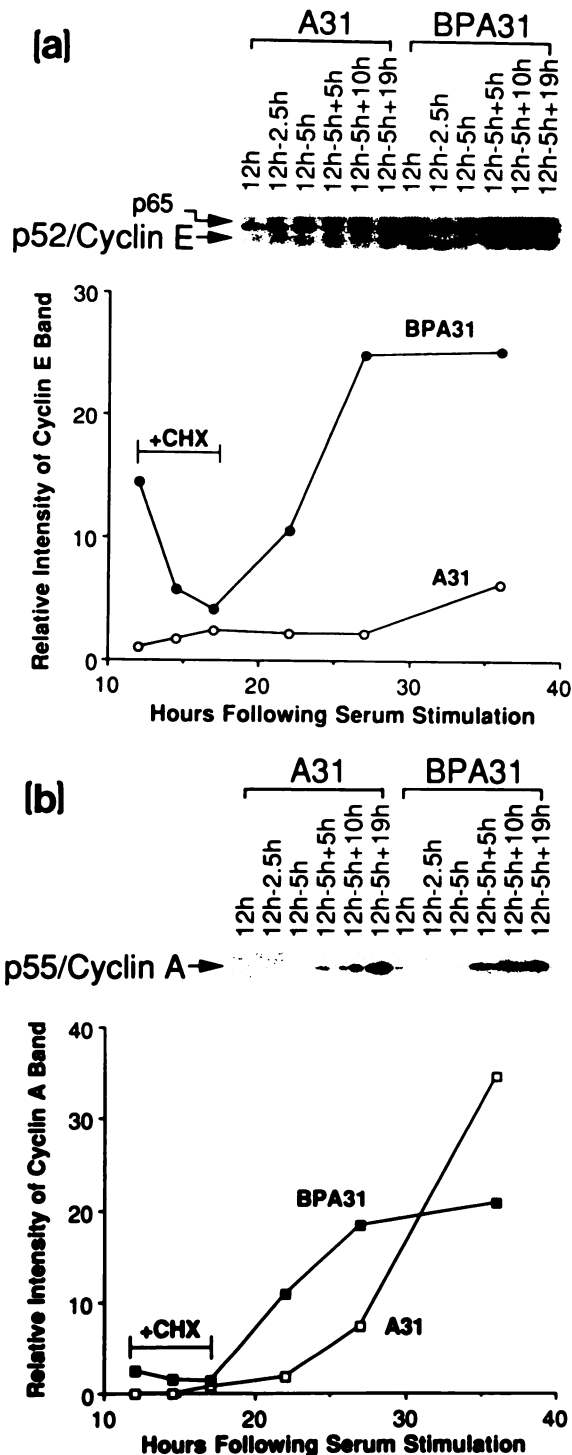

Fig. 4. Western blot analysis of cyclin E and cyclin A proteins. Nuclear extracts (40 μg/lane), prepared from both A31 and BPA31 cells during the CHX pulse chase process (see Fig. 3) at the indicated times in hours (*above lanes*), were assayed with anti-cyclin E (1:500) (*a*) and anti-cyclin A (1:300) (*b*). *Arrows*, p52/cyclin E, p65, and p55/cyclin A. The 12-h A31 sample was underloaded in this experiment.

higher in BPA31 than in A31 cells. Third, cyclin E protein recovered from the CHX inhibition much earlier in BPA31 than in A31 cells, probably because of its higher basal level (Fig. 4*a*). Although cyclin A in BPA31 and A31 cells was at a low level at 12 h (G_1/S) and during the CHX inhibition, it recovered to high levels about 5 hours earlier in BPA31 than in A31 cells (Fig. 4*b*). These differences were observed in multiple experiments. In contrast to these cyclins, *cdk2* protein in BPA31 and A31 cells was at a similar level and remained relatively unchanged during the pulse chase process (data not shown). These data suggest that the higher level and/or faster recovery of cyclin E/cyclin A proteins are probably responsible for the higher level and/or faster recovery of these *cdk* kinase activities in BPA31 cells.

By performing cell cycle and pulse chase experiments, we report here that accumulation of cyclin E and cyclin A proteins (Figs. 2, 4) and induction of their dependent kinases (Figs. 1 and 3) satisfy all three properties of the R protein (see "Introduction") and that therefore these kinases may be both proliferation controlling and deranged in these tumor cells. This idea is supported by several lines of experimental evidence. First, cyclin E, cyclin A, and *cdk2* have been suggested to be involved in the S phase of mammalian cells (5–10, 15–18). Second, constitutively expressed cyclin E or cyclin A overcomes the retinoblastoma protein-mediated suppression of proliferation (16). Third, this laboratory has recently found general cyclin (including A, B, and E) overexpression in several human breast tumor cell lines and a derangement in their order of appearance in synchronized tumor *versus* normal cells (19). Also, the aberrant expressions of cyclin A and cyclin D1 have been observed in some other cancers (reviewed in Refs. 5, 6, and 20). It is possible that transformed BPA31 cells, compared to A31 cells, might overexpress cyclin E and cyclin A genes, which are responsible for the higher basal level and/or faster recovery of cyclin E/cyclin A proteins (Fig. 4) that in turn up-regulate their dependent *cdk* kinase activities (Fig. 3). These properties probably determine reentry of these transformed cells into S phase with no extra delay and defective growth control (3). Taken together, these data suggest that cyclins may be a new class of protooncogenes that control cell proliferation and tumor growth.

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References

1. Pardee, A. B. A restriction point for control of normal animal cell proliferation. Proc. Natl. Acad. Sci. USA, *71:* 1286–1290, 1974.
2. Rossow, P. W., Riddle, V. G. H., and Pardee, A. B. Synthesis of labile, serum-dependent protein in early G1 controls animal cell growth. Proc. Natl. Acad. Sci. USA, *76:* 4446–4450, 1979.
3. Campisi, J., Medrano, E. E., Morreo, G., and Pardee, A. B. Restriction point control of cell growth by a labile protein: evidence for increased stability in transformed cells. Proc. Natl. Acad. Sci. USA, *79:* 436–440, 1982.
4. Pardee, A. B. G1 events and regulation of cell proliferation. Science (Washington DC), *246:* 603–608, 1989.
5. Hunter, T., and Pines, J. Cyclins and cancer. Cell, *66:* 1071–1074, 1991.
6. Pines, J. Cell proliferation and control. Curr. Opin. Cell. Biol., *4:* 144–148, 1992.
7. Reed, S. I. G1-specific cyclins: in search of an S-phase-promoting factor. Trends Genet., *7:* 95–99, 1991.
8. Pines, J., and Hunter, T. Human cyclin A is adenovirus-associated protein p60 and behaves differently from cyclin B. Nature (Lond.), *346:* 760–763, 1990.
9. Koff, A., Giordano, A., Desai, D., Yamashita, K., Harper, J. W., Elledge, S., Nishimoto, T., Morgan, D. O., Franza, B. R., and Roberts, J. M. Formation and activation of a cyclin E-*cdk2* complex during the G1 phase of the human cell cycle. Science (Washington DC), *257:* 1689–1694, 1992.
10. Dulic, V., Lees, E., and Reed, S. I. Association of human cyclin E with a periodic G1-S phase protein kinase. Science (Washington DC), *257:* 1958–1961, 1992.
11. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res., *11:* 1475–1489, 1983.
12. Jessus, C., Ducommun, B., and Beach, D. Direct activation of *cdc2* with phosphatase: identification of p13^{suc1}-sensitive and insensitive steps. FEBS Lett., *266:* 4–8, 1990.
13. Gautier, J., Norbury, C., Lohka, M., Nurse, P., and Maller, J. Purified maturation-promoting factor contains the product of a *Xenopus* homolog of the fission yeast cell cycle control gene *cdc2*$^+$. Cell, *54:* 433–439, 1988.
14. Dou, Q-P., Markell, P. J., and Pardee, A. B. Thymidine kinase transcription is regulated at G1/S phase by a complex that contains retinoblastoma-like protein and a *cdc2* kinase. Proc. Natl. Acad. Sci. USA, *89:* 3256–3260, 1992.
15. Fang, F., and Newport, J. W. Evidence that the G1-S and G2-M transitions are

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controlled by different *cdc2* proteins in higher eukaryotes. Cell, *66:* 731–742, 1991.
16. Hinds, P. W., Mittnacht, S., Dulic, V., Arnold, A., Reed, S. I., and Weinberg, R. A. Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. Cell, *70:* 993–1006, 1992.
17. Girard, F., Strausfeld, U., Fernandez, A., and Lamb, N. J. C. Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. Cell, *67:* 1–20, 1991.
18. Zindy, F., Lamas, E., Chenivesse, X., Sobczak, J., Wang, J., Fesquet, D., Henglein, B., and Brechot, C. Cyclin A is required in S phase in normal epithelial cells. Biochem. Biophys. Res. Commun., *182:* 1144–1154, 1992.
19. Keyomarsi, K., and Pardee, A. B. Redundant cyclin overexpression and gene amplification in breast cancer cells. Proc. Natl. Acad. Sci. USA, *90:* 1112–1116, 1993.
20. Jiang, W., Kahn, S. M., Tomita, N., Zhang, Y-J., Lu, S-H., and Weinstein, I. B. Amplification and expression of the human cyclin D gene in esophageal cancer. Cancer Res., *52:* 2980–2983, 1992.

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