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Novel Alterations in CDK1/Cyclin B1 Kinase Complex Formation Occur during the Acquisition of a Polyploid DNA Content

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The pathways that regulate the S-phase events associated with the control of DNA replication are poorly understood. The bone marrow megakaryocytes are unique in that they leave the diploid (2C) state to differentiate, synthesizing 4 to 64 times the normal DNA content within a single nucleus, a process known as endomitosis. Human erythroleukemia (HEL) cells model this process, becoming polyploid during phorbol diester-induced megakaryocyte differentiation. The mitotic arrest occurring in these polyploid cells involves novel alterations in the cdk1/cyclin B1 complex: a marked reduction in cdk1 protein levels, and an elevated and sustained expression of cyclin B1. Endomitotic cells thus lack cdk1/cyclin B1-associated H1-histone kinase activity. Constitutive overexpression of cdk1 in endomitotic cells failed to re-initiate normal mitotic events even though cdk1 was present in a 10-fold excess. This was due to an inability of cyclin-B1 to physically associate with cdk1. Nonetheless, endomitotic cyclin B1 possesses immunoprecipitable H1-histone kinase activity, and specifically translocates to the nucleus. We conclude that mitosis is abrogated during endomitosis due to the absence of cdk1 and the failure to form M-phase promoting factor, resulting in a disassociation of mitosis from the completion of S-phase. Further studies on cyclin and its interacting proteins should be informative in understanding endomitosis and cell cycle control.

INTRODUCTION

Two classes (or families) of proteins make up the protein-kinase complexes involved in the biochemical control of the cell cycle. The cell division kinases (CDKs), also referred to as cyclin-dependent kinases, are the catalytic subunits of these complexes, whereas the cyclins function as the regulatory subunits. Cyclins are proteins that undergo dramatic fluctuations in abundance as a function of cell cycle progression, and thus regulate the activation of the holoenzyme (Draetta and Beach, 1988; Draetta *et al.*, 1989; Murray *et al.*, 1989). Over the past few years, understanding of the biochemical control of the cell cycle has markedly improved, but is more complex than originally thought. For example, recent work demonstrated the existence of a family of related CDKs, each homologous to cdc2, the parent member of the CDK-family (cdc2 is also known as cdk1, which is the term used in this paper) (Meyerson *et al.*, 1992). Likewise, there are at least eight members of the cyclin gene family (Hunter and Pines, 1991; Matsushime *et al.*, 1991). Cyclin B1 is the best understood of the cyclins. It complexes with cdk1 to form M-phase promoting factor (MPF), the mitosis-initiating protein kinase complex (Pines and Hunter, 1989; Riabowol *et al.*, 1989). A- and E-type cyclins physically associate with cdk1 and/or other cell cycle kinases (e.g., cdk2), and function at both G_1/S and G_2/M transitions (Dulic *et al.*, 1992; Koff *et al.*, 1992; Pagano *et al.*, 1992). Finally, a family of G_1 cyclins (D cyclins in mammals and Cln in yeast) recently were identified that are important in timing G_1 progression as well as G_1/S transition (Lew *et al.*, 1991; Matsushime *et al.*, 1991; Xiong *et al.*, 1991).

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This new knowledge on the biochemical control of the cell cycle not withstanding, the pathways that regulate the S-phase events associated with the control of DNA replication, or the extent to which these pathways resemble those in lower organisms (e.g., yeast), remain poorly understood. In lower organisms, it is known that the length of S-phase varies at different developmental stages of the same species (Blumenthal *et al.*, 1974; Edenberg and Huberman, 1975; Hand, 1978). For example, *Xenopus* embryos have a S-phase of only 25 min compared with 10–12 h in adult cells (Callan, 1972). Similarly, in adult *Drosophila*, cells in vitro replicate their DNA during an S-phase of about 10 h, whereas it is less than 4 min in early embryonic cells (Blumenthal *et al.*, 1974). However, the exact mechanisms that regulate these differences in S-phase timing are unknown, as are similar control mechanisms in mammalian cells. Thus, the identification of a mammalian cell type with an inducible alteration in the cell cycle control that modulates S-phase would provide a useful model. Such a cell exists within the mammalian hematopoietic system; the bone marrow megakaryocyte, which is responsible for platelet formation, undergoes polyploidization in response to thrombopoietic demand.

The cellular hierarchy of the megakaryocytic lineage consists of three stages: progenitor cells, immature megakaryocytes (promegakaryoblasts), and mature megakaryocytes (for a review see Long and Hoffman, 1995). The megakaryocyte progenitor cells are responsible for the expansion of the megakaryocyte numbers and proliferate in a normal fashion in response to a number of mitotic growth factors. The promegakaryoblasts are transitional in nature, bridging the progenitor cells with the more mature, post-mitotic cells. These immature cells are not readily observed morphologically in vitro or in bone marrow specimens, but can be identified by their expression of megakaryocyte/platelet-specific markers (Jackson, 1973; Long and Williams, 1981b; Rabellino *et al.*, 1981). Promegakaryoblasts are quite restricted (or lacking) in their proliferative potential. They thus are the developmental stage at which megakaryocytes cease to proliferate but, uniquely, continue to acquire an increased DNA content during differentiation. As such, they are endomitotic (a mechanism of acquiring polyploid nuclei, see below) and contain an intermediate (6C→8C), DNA content (Long, unpublished observations; Young and Weiss, 1987). Mature megakaryocytes no longer proliferate, but approximately 25% continue to undergo endomitosis during maturation (see below).

Megakaryocytes are thus unique among mammalian marrow cells in that they leave the diploid (2C) state to differentiate, synthesizing 4 to 64 times the normal DNA content within a single nucleus (Odell *et al.*, 1970; Ebbe, 1976). The process of polyploidization occurs during the terminal differentiation of these cells (i.e., it is initiated after the proliferative phases of development, and precedes development of the earliest morphologically recognizable cell, the megakaryoblast; Long *et al.*, 1982b; Williams and Jackson, 1982). Thus, megakaryocyte cell cycle control is atypical in the sense of not having a typical 2C → 4C → mitosis progression. Nonetheless, the process of polyploidization is tightly regulated, as during each replicative event, the entire DNA content is duplicated. As a result, megakaryocytes contain multiples of the normal diploid DNA content (i.e., they are 4C, 8C, 16C, 32C, etc., where 2C is the normal DNA content of a cell in the G_0/G_1 phase of the cell cycle). In these cells, therefore, mitosis is disassociated from the completion of S-phase, but global control regarding the replication of DNA is retained.

The process of polyploidization is not, of course, unique to megakaryocytes, as certain plant, insect, and neoplastic cells also contain polyploid nuclei (Bennett, 1973; Therman *et al.*, 1983). Little is known concerning the mechanism by which cells achieve a polyploid DNA content. Polyploidization is defined as the acquisition of elevated DNA content by a cell, regardless of the mechanism by which such changes in ploidy occur. This process is mechanistically classified into the following: endomitosis, endoreduplication, and nuclear restitution (Therman *et al.*, 1983). Endoreduplication is a chromosome duplication cycle that results in polytenic insect cells, and nuclear restitution is a rare form of polyploidization that occurs when cells initiate, but do not complete, anaphase (Therman *et al.*, 1983). Endomitosis is the reproduction of nuclear elements not followed by chromosome movement or cytoplasmic division, and occurs in the presence of an intact nuclear membrane. Thus, endomitotic cells lack an anaphase and telophase. This definition best fits the process of polyploidization of megakaryocytes (Ebbe, 1976), although it is not known whether these cells retain an intact nuclear membrane.

Morphological observations of relatively mature megakaryocytes reveal that these cells do not progress through typical mitotic stages but, rather, undergo a type of "mitotic arrest." Clearly, some megakaryocytes reach metaphase as (endo)mitotic figures are seen in these cells, but the frequency of these figures is low, ranging from 0.3 to 0.8% (Jackson, 1990). Moreover, megakaryocytes do not seem to complete metaphase as anaphase events are never seen (Goyanes-Vuillaescusa, 1969; Ebbe, 1976; Radley and Green, 1989). Chromosome condensation occurs, but mitosis seems to halt somewhere between late prophase (Ebbe, 1976) and mid-metaphase, with the chromosomes forming a multifaceted metaphase plate (Radley and Green, 1989). Preliminary work has shown that some (complex) spindle fibers form (Goyanes-Vuillaescusa, 1969; Radley and Green, 1989), but their functional significance in endomitosis may be ques-

tioned, as treatment with vincristine does not increase the numbers of megakaryocytes in mitosis, as would be expected (Ebbe *et al.*, 1975). Interestingly, studies by Ebbe and Stohlman (1965) and Feinendegen *et al.* (1962) using tritiated thymidine pulse-chase experiments in vivo demonstrated that, although mature megakaryocytes do not take up this label, 25–40% of the earliest recognizable cells (i.e., megakaryoblasts) were synthesizing DNA, as they became labeled within a 30 min pulse. Similar values were determined by Odell *et al.* (1968) based on labeling index and generation time. Given that 50 times the number of megakaryocytes become labeled with tritium (compared to the numbers that show mitotic figures), it appears that these cells have dissociated M-phase from the completion of S-phase. However, with a prolonged tritium-exposure period, 100% of the megakaryocytes become labeled, indicating that majority of DNA synthesis actually occurs in a morphologically unrecognizable precursor cell (Feinendegen *et al.*, 1962; Ebbe and Stohlman, 1965). Work by Jackson (1973) and this laboratory (Long *et al.*, 1981a,b, 1982a,b) identified these immediate precursors as smaller (12–18 μ diameter) immature megakaryocytes (termed promegakaryoblasts), which as mentioned above, largely lack the ability to proliferate, but rather differentiate into mature cells while continuing to synthesize DNA (Long, unpublished observations; Young and Weiss, 1987).

The above morphological data, as well as the presence of a single, polyploid nucleus in each cell, suggest that cell cycle alterations occur such that the period of S-phase is prolonged, and DNA synthesis reiterated. They further imply that other changes must occur during the megakaryocytic M-phase to prevent nuclear division (e.g., anaphase and telophase occur late in M-phase). Unfortunately, the rarity of these cells and their precursors makes study of the biochemical control of polyploidization in primary cells technically difficult (Long *et al.*, 1990). We have developed a model system in which human erythroleukemia cells differentiate into early megakaryocytes (Long *et al.*, 1990). Thus, serum-free stimulation with low-dose (nanomolar) tumor-promoting phorbol diesters (e.g., phorbol myristate acetate; PMA) results in the commitment of human erythroleukemia (HEL) cells to the megakaryocyte lineage. This differentiation process drives the cells to an early phase of megakaryocyte development with the cells becoming morphologically and functionally similar to human megakaryoblasts. Morphological and ultrastructural changes occur that are consistent with that of an early megakaryocyte. As mentioned, one hallmark of megakaryocyte differentiation in these cells is the presence of a single nucleus containing a 4- to 32-fold increase in DNA content. A coordinate relationship between the expression of megakaryocyte antigens (as a marker of differentiation) and an increase in DNA content in HEL cells was noted, with those cells having the highest antigen expression also having the highest DNA content (Long *et al.*, 1990). Stimulation with nanomolar PMA shifts the majority of the cells (i.e., those in G_0/G_1) into DNA synthesis. Phorbol-treated HEL cells thus go through successive twofold increases in DNA content reaching levels approximately 4 to 16 times that of their resting DNA content (within a single nucleus) within a 3–7 day period. It is important to note that cellular proliferation ceases within the first 24 h of PMA stimulation, but DNA synthesis continues for 7 days (Long *et al.*, 1990). Thus, these cells (like normal megakaryocytes) undergo a mitotic arrest in that they abrogate mitosis, fail to reach anaphase, and retain an elevated DNA content within a single nucleus. By approximating the developmental program of normal megakaryocytes, HEL cells are an important model for examining the mechanism(s) of polyploidization. This study was undertaken to specifically address involvement of cell cycle–associated proteins and protein kinase complexes in the mitotic arrest associated with the acquisition of a polyploid DNA content. These results demonstrate the absence of a functional cdk1/cyclin B1 protein kinase complex in endomitotic cells, and a putative role for cyclin B1 in the process of polyploidization.

MATERIALS AND METHODS

Induction of Endomitosis and Cellular Subcloning

HEL cells were passaged and induced to undergo endomitosis as described (Long *et al.*, 1990). Briefly, HEL cells were grown to a density of 10^6 cells/ml, washed twice in phosphate-buffered saline (PBS), and induced at 5×10^4 to 1.0×10^5 cells/ml in RPM1 1640 containing 0.5% fetal calf serum, in the presence (or absence as control) of 2 nM 4β phorbol 12-myristate 13-acetate (PMA). To enhance their endomitotic potential, HEL cells were subcloned by limiting dilution and screened for inducible increases in DNA content by flow cytometry. Of 30 single cell clones, one demonstrated a higher percentage of cells in endomitosis. This subclone (HEL-023) was used for all the experiments described herein. To address the questions of endomitosis-specific effects, a PMA-resistant subclone (PMA_r) of HEL-023 cells was developed. This clone was generated by cultivating HEL-023 cells in the presence of 2 nM PMA (added weekly with the addition of fresh media) until a proliferating PMA-resistant cell population was generated (approximately 8 mo). These PMA_r cells were next subjected to two rounds of single cell cloning (by limiting dilution). The resulting clone has been stable for over 3 yr. When studied as a control, PMA_r cells were stimulated with a 100-fold excess of PMA (i.e., 2×10^{-7} M).

Flow Cytometric Analysis

Cell samples containing 10^6 cells were fixed by gradual addition of ice-cold methanol while mixing, incubated 30 min on ice, and centrifuged for 5 min at $300 \times g$. The supernatant was discarded and cell pellets were stained with 1 ml of propidium iodide solution (100 μg/ml propidium iodide in 0.1% Triton X-100 and .0037% EDTA). Each sample was treated at room temperature with 100 U/ml RNase (Sigma Chemical Co., St. Louis, MO). DNA content was measured on 20,000–40,000 cell nuclei using a Coulter Epics Elite

flow cytometer (Hialeh, FL). DNA histograms and the cell cycle analysis of the G_0/G_1, S, and G_2/M phases, as well as the DNA content of each ploidy class, were determined with the Elite Software Package.

Immunological Reagents

Polyclonal antibodies to cdk1 and cyclin B1 were generous gifts of Drs. D. Beach, Cold Spring Harbor Laboratory (cdk1), D. Fraser, NCI Frederick (cdk1), and T. Hunter, Salk Institute (cyclin B1). Similarly, cdk2 and cdk1 antibodies were obtained from UBI, Lake Placid, NY, whereas monoclonal antibodies to cyclin B1 were purchased from PharMingen, San Diego, CA.

Counter-Flow Centrifugal Elutriation

Control cells and endomitotic cells were separated into cell cycle–specific phases (G_0/G_1, S, and G_2/M for control cells, and 2C, 4C, 8C, and 8C/16C for endomitotic populations) by counter-current centrifugal elutriation using a Beckman JE6B elutriation rotor. Endomitotic cells were isolated 5–7 days after induction with 2 nM PMA. Control cells were incubated in identical conditions without PMA (i.e., uninduced controls), whereas PMA_r cells were incubated with 10^{-7} M PMA (hereinafter "control cells" refers to both uninduced HEL cells and PMA_r cells). Between $3–4 \times 10^8$ cells in 5 ml of elutriation buffer (PBS/0.3 mM EGTA/0.1% BSA/0.1% glucose) were loaded into a standard elutriation chamber at a flow rate of 10–12 ml/min with the rotor spinning at 2200 rpm. Fractions of 100 ml were collected at each flow rate, and the flow increased in increments of 3–4 ml/min from 20 ml/min to 80 ml/min. All fractions were kept on ice and aliquots were removed for DNA content analysis. Elutriated cells were washed with PBS, centrifuged, and the cell pellets were frozen at −70°C until used.

SDS-PAGE and Western Analysis

For Western analysis, cells were washed twice with cold PBS and lysed for 30 min at 4°C in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 20 mM EDTA, 50 mM NaF, 1% NP-40, 0.02% NaN_3) containing 10 μg/ml of the protease inhibitors aprotinin, leupeptin, trypsin inhibitor, and 1 mM of the inhibitors sodium orthovanadate, phenylmethylsulfonyl fluoride, and tosylphenylalanine chloromethyl ketone. Cell lysates were cleared by centrifugation at 14,000 × *g* for 45 min. An aliquot of each lysate was removed for protein concentration determinations. SDS-PAGE was performed in 10–12% polyacrylamide (Laemmli, 1970). Each lane contained between 40 and 80 μg of total cell (lysate) protein. For a given Western analysis, each lane received equal protein loads. Prestained molecular weight standards were run in parallel lanes. After electrophoresis, the proteins were transferred to nitrocellulose paper in buffer containing 25 mM Tris-HCl, 192 mM glycine, 20% v/v methanol, 0.01% SDS (pH 8.5). Residual protein binding sites on the nitrocellulose were blocked by incubation for 3 h to overnight in TBST buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.5% Tween-20) containing 5% nonfat dry milk. The nitrocellulose was then incubated with primary antibody for 2 h to overnight. After washing with TBST, the filter was incubated with the second antibody (anti-IgG conjugated with horseradish peroxidase) for 20–30 min. Finally, the proteins were visualized by autoradiography using the ECL Western analysis detection reagents (Amersham, Arlington Heights, IL). The H1 histone kinase activities and protein band intensities on ECL Western autoradiograms (all with exposures within the linear range of the film) of immunoprecipitated proteins were quantitated using a scanning laser densitometer (Ultroscan XL, LKB, Gaithersburg, MD).

Immunoprecipitation and Kinase Assays

Cellular extracts were preadsorbed with either rabbit or mouse IgG-agarose (Sigma), and the adsorbed lysates were next incubated with either polyclonal or monoclonal antibodies for 16–18 h at 4°C. Antigen-antibody complexes were pelleted using either protein A-agarose (Sigma) or protein G-sepharose (Pharmacia, Piscataway, NJ) after incubating for 1 h with constant rotation. Immunoprecipitates were washed three to five times with a buffer containing 50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 1 mM EDTA, 0.25% gelatin, 0.02% NaN_3, 0.1–0.5% NP-40 followed by 10 mM Tris-HCl, pH 7.4, 0.5% NP-40, and protease inhibitors, resuspended in SDS-PAGE sample buffer, and heated to 95°C for 5 min. Proteins were resolved in SDS-PAGE as described above. Control precipitations were performed using 5 μl of normal rabbit serum.

For in vitro protein kinase assays, immunoprecipitates were washed three times with lysis buffer, followed by two washes with kinase buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 mg/ml bovine serum albumin). Washed immunoprecipitates were then combined with kinase assay mixture containing 1 μg histone H1 (Boehringer Mannheim Biochemicals, Indianapolis, IN), 30 μM [γ-^{32}P]ATP (5 μCi) in a final volume of 50 μl. After 30 min of incubation at 30°C, the reaction products were analyzed by SDS-PAGE.

Pulse-Chase Experiments

Normally proliferating and endomitotic HEL cell cultures were labeled for 1 h in methionine-free RPMI media containing 100 μCi/ml of Trans-label (ICN, Costa Mesa, CA). The medium was then changed to complete RPMI media plus 0.5% fetal calf serum, and individual flasks/dishes were harvested at 1, 2, 3, 4, 8, 12, 16, and 24 h. The labeled proteins were immunoprecipitated with antibody against cyclin B1 or cdk1 and processed as above. The dried gels were then exposed to autoradiography and the labeled bands were quantitated using a Quick Scan R&D Electophoresis/TLC densitometer (Helena Labs, Beaumont, TX).

Expression and Selection of Recombinant Human cdk1

A human cdk1 hs cDNA cloned into vector pOB231 was a generous gift from Paul Nurse (Imperial Cancer Research Fund, Oxford, England). This contained a 2-kb cDNA insert of cdk1, corresponding to the open reading frame of cdk1. This cDNA was cloned into pCEP4 (Invitrogen, San Diego, CA), an episomal expression vector, containing the hygromycin B resistance gene. HEL cells were transfected by electroporation using vectors containing cdk1 hs cDNA either in sense or antisense orientation and the vector only (as control). Electroporated cells were selected in hygromycin B (350 μg/ml) containing medium. Clones containing antisense cdk1 did not show any change in cdk1 protein expression compared to vector only control. Further single-cell clones of the overexpressed cells were isolated by limiting dilution to obtain clones with maximum expression of this protein and were chosen to be used in the subsequent experiments. The accumulation of cdk1 protein in these cell lines was variable. Our unpublished densitometric observations show that cdk1 levels ranged from approximately that of wild-type levels to an approximate 10-fold increase (see RESULTS).

RESULTS

Polyploidy in Human Erythroleukemia Cells

We previously demonstrated that HEL cells undergo endomitosis during phorbol diester-induced megakaryocyte differentiation (Long *et al.*, 1990). As illustrated in Figure 1, this process is associated with the acquisition of an increased (polyploid) DNA content within a single nucleus (Figure 1A). To enhance their endomitotic potential, these HEL cells were subcloned

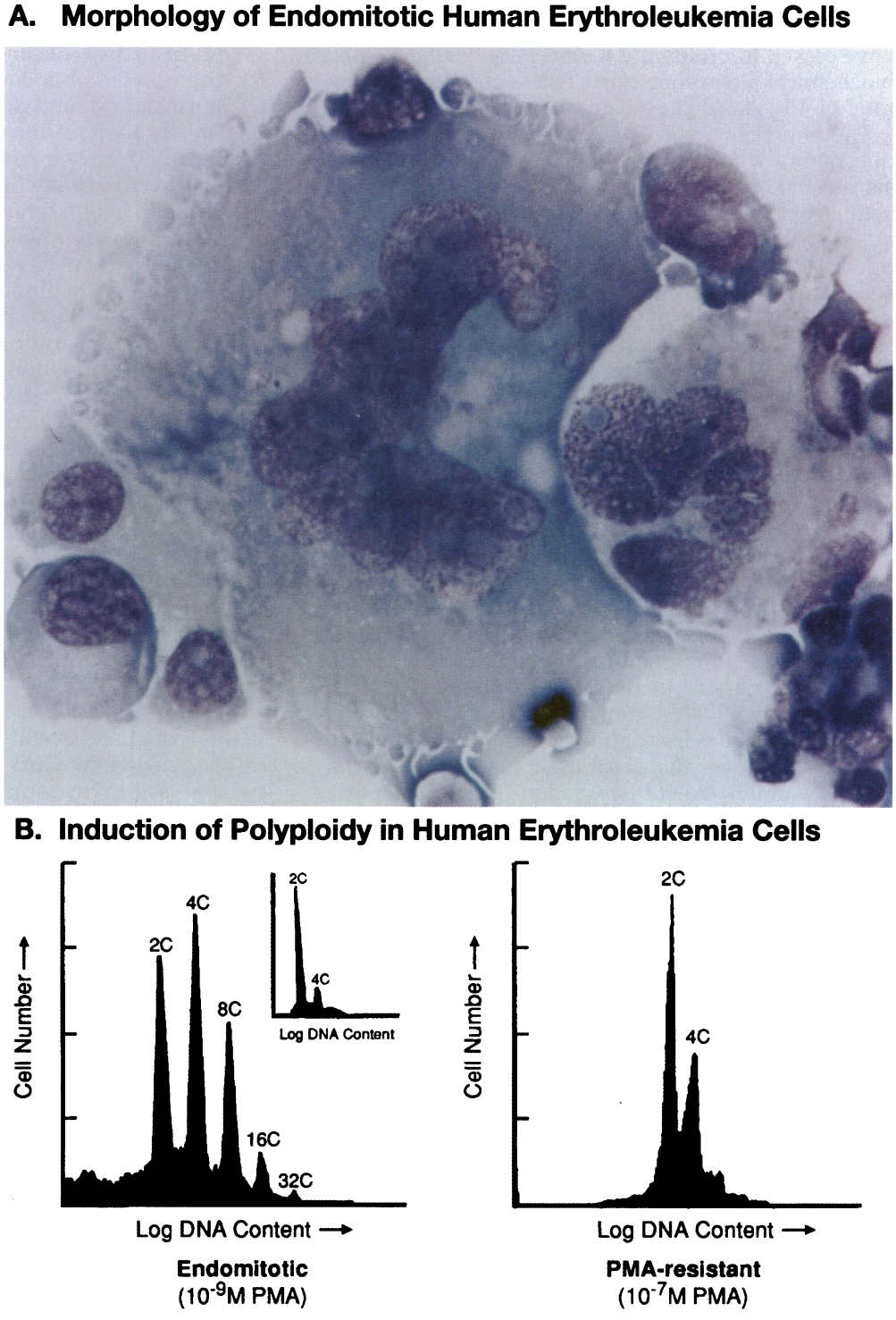

Figure 1. Morphology and DNA content of polyploid human erythroleukemia (HEL) cells. HEL cells were induced for 5 days with PMA and cultured as described in MATERIALS AND METHODS. (A) A cytocentrifuged preparation stained with May-Gruenwald Giemsa. The large (≈ 50μ in diameter) central cell and the smaller (≈25μ diameter) cell to the right show the lobulated nucleus seen in these polyploid cells. (B) Histograms of control (i.e., uninduced; inset), PMA-treated (left panel), and PMA-resistant cells (right panel) DNA content. 2C, the chromosome content of diploid cells, the DNA content of a cell in the G_0/G_1 phase of the cell cycle. Histograms are of 20,000 isolated nuclei stained with propidium iodide. PMA, 4-β phorbol 12-myristate 13-acetate.

by limiting dilution, PMA induced, and screened for increased levels of polyploidy by flow cytometric analysis. Of these subclones, one (HEL-023) demonstrates a high percentage of cells in endomitosis following induction with 2 nM PMA (Figure 1B, left panel). It should be realized that induced cells possessing a 2C/4C DNA content (where 2C is the DNA content of a G_0/G_1 cell) represent cells in the early

phases of endomitosis. Although this distinction cannot be made on the basis of DNA content, these cells have ceased to proliferate (Long *et al.*, 1990) and show biochemical alterations consistent with other endomitotic ploidy classes (see below).

The phorbol responsiveness of HEL cells raises the question of whether a given observation is endomitosis specific or a trivial effect of the known pleiotropic actions of tumor-promoting phorbol diesters (Sibley *et al.*, 1984; Yoshimasa *et al.*, 1987). We therefore developed a PMA-resistant sub-clone (PMA_r) of HEL-023 cells. This clone was generated by continuous culture of HEL-023 cells in the presence of 2 nM PMA, followed by two rounds of single-cell cloning, again in the presence of PMA. The resultant PMA_r clone has been stable for over 3 yr, and is passaged in the presence of 2 nM PMA. Importantly, PMA_r cells do not undergo endomitosis even at 100-fold excess PMA (10^{-7} M, Figure 1B, right panel).

Cell Cycle Separation of Proliferating, Endomitotic, and PMA-Resistant Cell Subpopulations

To study the biochemical mechanisms associated with endomitosis, we separated control HEL cells into cell cycle–specific subpopulations using counter-flow centrifugal elutriation. In this way, control cells of 2C, S-phase, and 4C DNA content can be compared with endomitotic cells. Elutriation of uninduced (control) HEL cells allows the separation of discrete G_0/G_1, S-phase, and G_2/M subpopulations of 80–90% purity (Figure 2A). PMA_r cells were also separated into G_0/G_1, S-phase, and G_2/M content by the same methodology resulting in equivalent purities. Elutriation of endomitotic cells separates discrete populations of 2C, 4C, 8C, and 16C DNA content, averaging ~70% purity (Figure 2B). This slight reduction in resolution was expected as we previously demonstrated that these ploidy classes are not associated with exact cell-size increments (Long *et al.*, 1990). Nonetheless, the resolution of endomitotic subpopulations allows detection of endomitosis-associated alterations in cell cycle control proteins.

Endomitosis Is Associated with Alterations in the Relative Abundance of cdk1 and Cyclin B1 Proteins

We hypothesized that one component of the acquisition of a polyploid DNA content is the abrogation of mitosis, resulting in a uninucleate cell. The biochemical correlate of this is that some alteration(s) should occur in the highly conserved cdk1/cyclin B1 protein kinase complex that drives mitosis (Arion *et al.*, 1988; Draetta and Beach, 1988; Riabowol *et al.*, 1989). To test whether cyclin B1 and cdk1 play a role in endomitotic HEL cells, we determined the relative abundance of these proteins, as well as the H1-histone kinase activity of the cdk1/cyclin B1 complex, in normally proliferating and endomitotic HEL cells.

Western analysis of control cell lysates demonstrates that the relative abundance of cdk1 in proliferating (i.e., normally cycling) and PMA_r cells remains fairly constant throughout the cell cycle (Figure 3A, upper row, left and center panels). In sharp contrast, endomitotic cells contain markedly reduced levels of cdk1 protein. In unfractionated endomitotic cells, cdk1 is observed at very low levels (Figure 3A, upper row, right panel), whereas in elutriated 2C, 4C, and 8C cells, it is below the limits of detection by ECL-based Western analysis (Figure 3, upper row, right panel).

We next examined the relative abundance of cyclin B1, as this protein is the "classic" mitotic cyclin that binds with cdk1 (Draetta and Beach, 1988; Pines and Hunter, 1989). Western analysis of cyclin B1 levels in control cells demonstrates the expected periodicity of this protein in normally proliferating and PMA_r cells (Figure 3A, lower row, left and middle panels). Unexpectedly, the relative abundance of cyclin B1 is increased in 2C endomitotic cells when compared with control 2C cells (Figure 3A, lower row, right panel). Endomitotic cells also fail to show the expected cyclin B1 periodicity as the relative abundance of this protein remains constant in each of the subsequent ploidy classes.

To determine whether a functional interaction exists between cdk1 and cyclin B1, we examined their physical association in vitro. Protein complex formation was assessed by measuring the presence of cyclin B1 in anti-cdk1 immunoprecipitates. Analysis of the immunoprecipitates of control and endomitotic cell lysates demonstrates that cdk1 physically associates with cyclin B1 in control cells, while this association is greatly reduced in endomitotic cells (Figure 3B, left panel). The function of these cyclin B1 complexes was determined in H1 histone kinase assays. As expected, uninduced control cells show high levels of cdk1-associated H1-histone kinase activity. In sharp contrast, there is little detectable cdk1/cyclin B1 H1-histone kinase activity in endomitotic cells (Figure 3B, right panel) even though immunoprecipitation shows that some cyclin B1 remains associated with cdk1 in endomitotic cells. Consistent with this, normalization of the histone-kinase activity to the amount of cdk1 precipitated demonstrates that endomitotic cells show a 70% reduction in cdk1-associated kinase activity (Table 1A).

The mechanism of cyclin B1 elevation in endomitotic cells was determined by analysis of both cyclin B1 mRNA levels as well as its protein half-life. Northern analysis demonstrates that cyclin B1 mRNA undergoes a sixfold elevation following the induction of endomitosis (Figure 4A). Additionally, pulse-chase analysis of cyclin B1 protein shows that it is stabilized in polyploid cells with protein half-life changing from

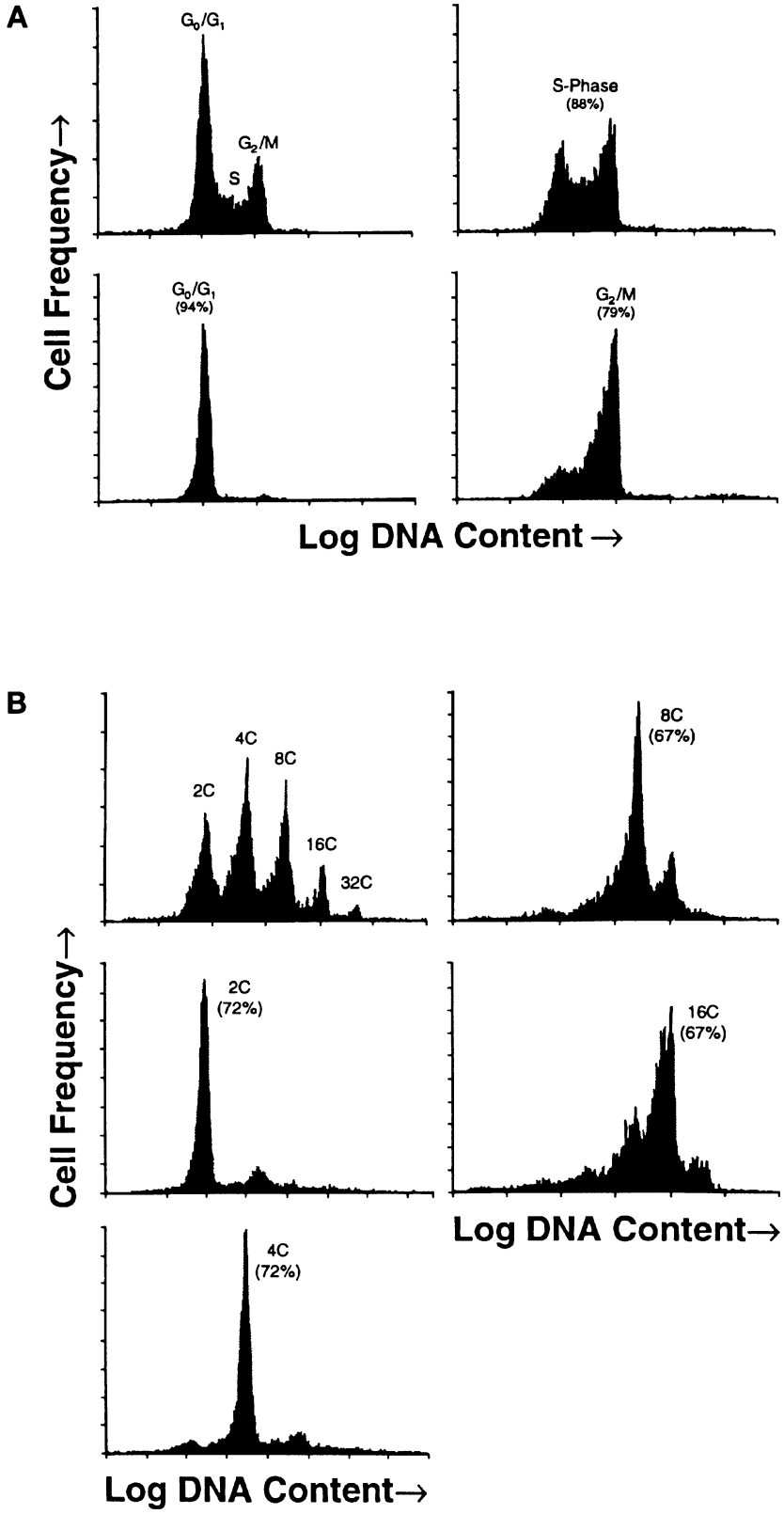

Figure 2. DNA content of elutriated cycling and endomitotic cells. Counter-flow elutriations performed as described in MATERIALS AND METHODS. Percents in parentheses are purities of the elutriated populations.

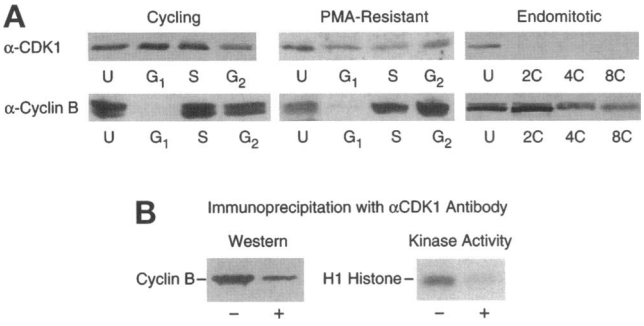

Figure 3. Relative protein abundance, physical association, and H1-histone kinase activity of cdk1 and cyclin B1 in endomitotic cell populations. Western analysis of whole cell lysates performed as in MATERIALS AND METHODS and immuno-blotted proteins visualized by enhanced chemiluminescence (ECL)-based Western analysis. U, unfractionated cells (i.e., elutriation input); G_1, S, and G_2, cell cycle phases; 2C, 4C, and 8C, ploidy classes. (A) ECL-based Western analysis of cdk1 and cyclin B1. (B) Physical association of cdk1 and cyclin B1. Control (−) and endomitotic (+) cells were immunoprecipitated with antibody to cdk1 and probed by ECL Western analysis using antibody specific to cyclin B1. Parallel immunoprecipitates were used in H1-histone kinase assays (see MATERIALS AND METHODS). + and −, the presence or absence (respectively) of the endomitosis-inducing agent PMA.

Table 1. Normalization of cdk1 and cyclin B kinase activities

A. Whole cell lysates

IP Antibody	Normalized kinase activity[a] (specific activity × 10^{-2})[b] Control	Endomitotic	Percent Change[c]
α-cdk1	35.0	11.0	−69.0
α-Cyclin B in cdk1 transfectants			
Vector control	17.0	14.0	−17.7
Cdk1 transfected	22.0	14.0	−22.0

B. Following immune depletion of cdk1

Condition	Total kinase activity[a,d]	Total cdk1 precipitated	Specific activity
Pre-immune depletion	883	9.5	93
Post-immune depletion	63	0.63	100

C. Following cyclin B1 immunoprecipitation of cdk-1–depleted extracts

Condition	Total kinase activity[a]	Total cyclin B1 precipitated	Specific activity
Control	31	1.85	17
Endomitotic	250	2.3	109

Normalized values determined as described in MATERIALS AND METHODS.

[a] Kinase activities are for H1-histone phosphorylation as described in METHODS.

[b] Specific activities are calculated as total kinase activity divided by the total amount of precipitated protein.

[c] Percent change is calculated as the increase or decrease in specific activity divided by the baseline value × 100.

[d] Immune depletion values are for control HEL cells. All values are in arbitrary units.

12 h in the control cells to 30 h in endomitotic cells (Figure 4B). In contrast, cdk1 half-life in endomitotic cells is reduced from 9 h (in controls) to 2 h (Figure 4C). These data demonstrate that the cdk1/cyclin B1 protein kinase complex functions normally in control HEL cells, with cdk1 concentrations staying constant throughout the cell cycle, whereas cyclin B1 is elevated in G2/M and then degraded in G_0/G_1 to release the cells from mitosis. In sharp contrast, endomitotic cells contain little detectable cdk1/cyclin B1 kinase activity due, not to the loss of cyclin B1, but rather to the absence of the apoenzyme cdk1. Interestingly, cyclin B1 fails to show the expected periodicity in endomitotic cells, and is both elevated and sustained, suggesting a role for this cyclin in establishing polyploidy.

Overexpression of cdk1 Fails to Inhibit Endomitosis

To determine the functional consequences of cdk1 reduction in endomitosis, cdk1 was constitutively overexpressed in HEL cells. Stable cdk1-HEL cell transfectants were isolated that expressed an approximate 10-fold increase in cdk1 levels compared with mock-transfected (vector only) controls (Figure 5A, upper panel, left). As expected, vector controls show a reduction in cdk1 expression when PMA is used to induce endomitosis. In contrast, cdk1-overexpressing HEL cells fail to show a reduction in the relative abundance of cdk1 following PMA-induction (Figure 5A, upper panel, right). Determination of the DNA content of these transfectants demonstrates that the simple process of reconstituting cdk1 levels in endomitotic cells was not sufficient to block the formation of polyploid nuclei (Figure 5A, lower panels).

Two alternative explanations exist for the failure of cdk1 overexpression to inhibit endomitosis. One is that the reduction in cdk1 protein abundance is neither necessary nor sufficient to cause polyploidy. Alternatively, cdk1 overexpression may occur, but fail to reconstitute the cdk1/cyclin B1 complexes. We explored this possibility by examining the physical association of cdk1 and cyclin B1 in the cdk1-transfected cells. Immunoprecipitation of cdk1-transfected cells with anti-cyclin B1 demonstrates that cdk1 co-precipitates with cyclin B1 in uninduced cells (i.e., in both vector-controls and cdk1-overexpressing cells; Figure

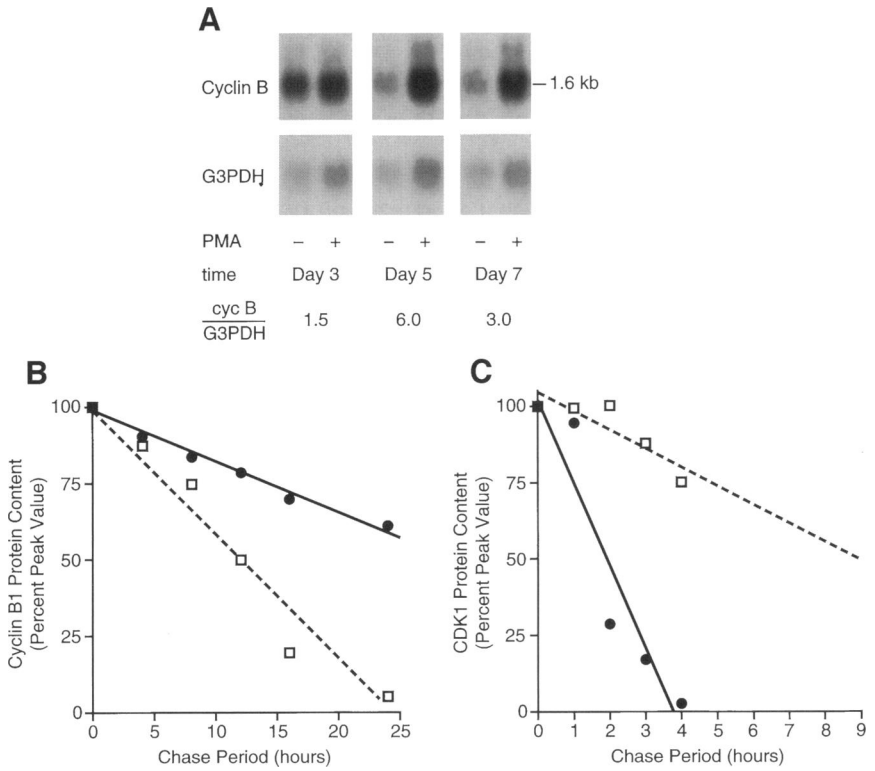

Figure 4. Relative mRNA abundance and protein half-life of cyclin B1 and cdk1. (A) Relative abundance of cyclin B1 mRNA. Upper panels, cyclin B1 mRNA; lower panels, glyceraldehyde 3 phosphodehydrogenase (G3PDH) mRNA, cycB/G3PDH represents cyclin B mRNA normalized to G3PDH. PMA, 4β-phorbol 12-myristate 13-acetate. (B) Pulse-chase analysis of cyclin B1 protein half-life. (C) Pulse-chase analysis of cdk1 protein half-life. In half-life studies, cells were metabolically labeled with [^{35}S]methionine, and cyclin B1 was "chased" for varying periods by immunoprecipitation and visualization by autoradiography. Autoradiograms (within the linear region of the film) were quantitated by densitometry. Solid lines/closed symbols, endomitotic cells; dashed lines/open symbols, control cells.

5B, upper panel). Thus, cdk1 and cyclin B1 are physically associated in both normally proliferating, cdk1-transfected cells and in vector controls. As expected, cdk1 fails to co-precipitate with cyclin B1 in PMA-treated cells transfected with vector only, due to the reduction in cdk1 relative abundance associated with endomitosis. However, anti-cyclin B1 immunoprecipitation of endomitotic, cdk1-overexpressing cells demonstrates that very little cdk1 is associated with cyclin B1, even though the cells are expressing approximately 10 times as much cdk1 as the vector controls. We conclude that cdk1 overexpression fails to block endomitosis due to a reduced or absent capacity of endomitotic cyclin B1 to physically associate with cdk1. Thus, while cdk1 relative abundance is high, the amount of cyclin B1 physically associated with cdk1 remains low in endomitotic cells.

Immune Depletion of cdk1 Fails to Abolish Endomitotic Cyclin B1-associated Histone Kinase Activity

A functional role for cyclin B1 in endomitosis was suggested in cyclin B1-associated H1 histone kinase assays, in which we examined the kinase activity of cyclin B1 immunoprecipitates in cdk1-transfected cells. These data showed that, in uninduced vector controls, cyclin B1 immunoprecipitates contained H1 histone kinase activity (Figure 5B, lower panel), as would be predicted given the co-precipitating presence of the apoenzyme cdk1 (Figure 5B, upper panel). Interestingly, a cyclin B1–associated kinase activity is observed in both PMA-treated vector-controls and PMA-treated cdk1 transfectants, which is not consistent with the endomitosis-associated reduction in cdk1 levels in these cells. Moreover, the amount of cyclin B–associated H1 histone kinase activity is equivalent in both vector controls or cdk1 transfectants, despite the 10-fold overexpression in the latter cells. This is true even when the cyclin B1–associated H1 histone kinase activity is normalized for the amount of protein immunoprecipitated (Table 1A). These data imply that cdk1 can be overexpressed in the presence of cyclin B1 without inducing kinase activity. However, the mechanism for this lack of increase in H1 histone kinase activity differs in control and endomitotic cells. Given a 1:1 stoichiometry between cyclin B and cdk1 in the formation of the holoenzyme (Draetta and Beach, 1988), it is not surprising that control cells do not show increased cyclin B–associated kinase activity, as the majority of the excess cdk1 in these cells is presumably free. In contrast, endomitotic cells show elevated levels of cyclin B (Figure 3), but do not show an increase in cdk1-associated kinase activity in cdk1 transfectants. The reason for this appears to be the lack of a physical association between cyclin B1 and cdk1 in these cells (Figure 5B, upper panel). Nonetheless, en-

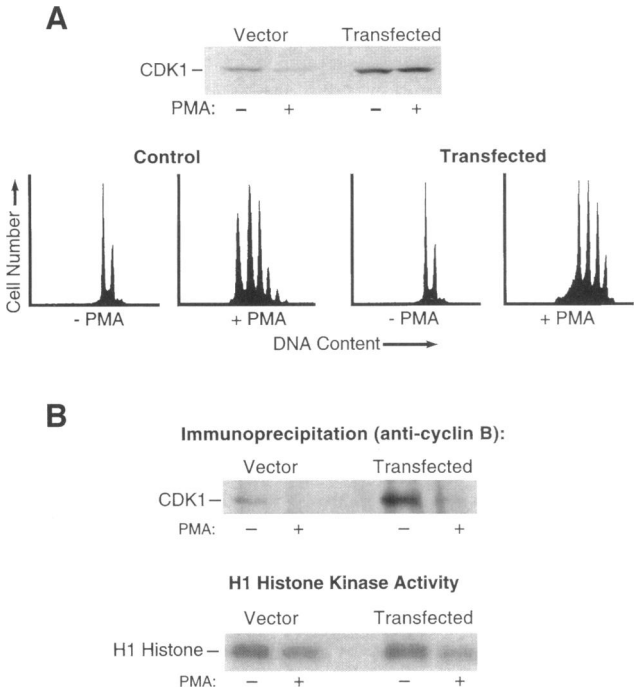

Figure 5. Overexpression of cdk1 in endomitotic cells. HEL cells were transfected with the pCEP4 vector containing the full-length human cdk1 cDNA or vector alone (see MATERIALS AND METHODS). cdk1 transfectants showed an approximate 10-fold increase in cdk1 expression. (A) Overexpression of cdk1 fails to inhibit endomitosis. Upper panel, relative abundance of cdk1 protein in transfected cells; lower panel, DNA histograms of cells transfected with vector only (control) and cdk1 (transfected). Clonal variation among cdk1 overexpressing cells was excluded by isolating 12 cdk1 overexpressing single-cell clones, none of which show a diminuation of endomitosis. (B) Physical association of cdk1 and cyclin B does not occur in cdk1-transfected cells. Transfected cells were immunoprecipitated with an antibody to cyclin B1 and immunoprecipitates were analyzed for the presence of cdk1 by ECL-Western (upper panels). Parallel aliquots were analyzed for H1-histone kinase activity (lower panels). Abbreviations are as in Figure 3.

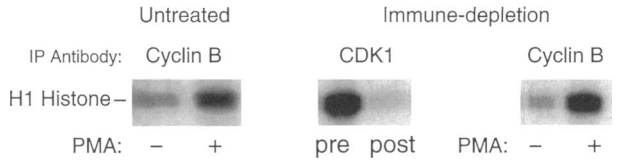

Figure 6. Cyclin B1–associated histone kinase activity. The failure of cdk1-immune depletion to abolish endomitotic cyclin B1–associated kinase activity was demonstrated as follows. Control (PMA−) and endomitotic (PMA+) cell lysates were immunoprecipitated with antibody to cyclin B1, and precipitates were analyzed for H1-histone kinase activity (left panel). These assays indicate that cyclin B1 precipitates an associated kinase activity even though the lysates contain very little detectable cdk1-associated kinase activity (Figure 3, bottom panel). To confirm that the cyclin B1–associated kinase activity was not due to residual cdk1, cell lysates were exhaustively depleted (3×) of cdk1. Analysis of control lysates show that two rounds of immune depletion markedly reduces (>95%) cdk1 histone kinase activity (center panel). Parallel depletions were performed on endomitotic cell lysates even though these cells have very little cdk1 to start with (Figure 3A, upper panel). These immune depleted lysates were then immunoprecipitated with anti-cyclin B1, and the precipitates were evaluated for H1 histone kinase activity (right panel).

domitotic cells (both vector and transfected) do contain a cyclin B–associated H1 histone kinase activity.

The surprising observation that a cyclin B1–associated kinase activity exists in the relative absence of cdk1 in transfected, endomitotic cells was confirmed in a series of immune-depletion experiments. Endomitotic cyclin B1–associated histone kinase activity, in the absence of detectable cdk1, was also observed in nontransfected endomitotic cells (Figure 6, left panel). To ensure that this activity was not due to residual (but undetectable) cdk1 associated with cyclin B1, control and endomitotic cell lysates were subjected to three rounds of immune depletion using anti-cdk1 antibody. Following two rounds of immune depletion, control lysates show a ≈95% reduction in total cdk1-associated H1 histone kinase activity without alterations in the specific activity of the cdk1 kinase complex (Figure 6, center panel, and Table 1B). Endomitotic cell lysates (which lack detectable cdk1 before immune depletion), were also subjected to three rounds of immune depletion. The supernatants from these immune depletions of control and endomitotic cells were then immunoprecipitated with anti-cyclin B1 and tested for H1 histone kinase activity. These data show that lysates from endomitotic cells, exhaustively depleted for cdk1, retain high levels of cyclin B1–associated H1 histone kinase activity (Figure 6, right panel). Indeed, data normalized for the amount of cyclin B immunoprecipitated indicates a sevenfold increase in total cyclin B–associated kinase activity in the absence of detectable cdk1 (Table 1B). However, this change in total cyclin B–associated kinase activity is due to, in part, a fivefold increase in the specific activity of the cyclin B kinase complex. This strongly suggests that cyclin B associates with different regulatory proteins during endomitosis. Confirming this, unpublished observations of silver-stained cyclin B immunoprecipitates resolved by SDS-PAGE show the presence of a new 90- to 100-kDa protein physically associated with cyclin B in endomitotic cells. It was also noted that cdk1-depleted control cell lysates possessed low amounts of H1-histone kinase activity. However, it cannot be excluded that this is not due to the residual cdk1-associated activity remaining following immune depletion. We next used ECL-based Western analysis to evaluate cyclin B1 immunoprecipitants (from cdk1-depleted cytoplasms) to determine whether other CDKs were associated with cyclin B1 in endomitotic cells. These unpublished data demonstrate that endomitotic cyclin B1 is not associated with PSTAIR-containing CDKs (i.e., cdk1, 2, or 3), nor

is it associated with cdk4 when it is evaluated by Western analysis using an anti-cdk4 antibody .

Nuclear Translocation of Endomitotic Cyclin B1

As B-type cyclins are cytoplasmic and translocate to the nucleus at the onset of mitosis (Pines and Hunter, 1990; Gallant and Nigg, 1992), we employed immunocytochemical localization to evaluate this process in control and endomitotic cells. Evaluation of control cells shows that approximately 20% of these cells contain detectable levels of cyclin B1 that localized to the cytoplasm (Figure 7A), which is consistent with the percentage of HEL cells in the G2/M phase of the cell cycle. Following induction of endomitosis, 100% of the endomitotic cells show the presence of nuclear cyclin B1 (Figure 7B). In addition, a subpopulation of endomitotic cells is observed that contains both cytoplasmic and nuclear cyclin B1. The functional significance of this subpopulation is not clear; however, unpublished results show that these cytoplasm-positive cells do not co-segregate with distinct ploidy classes in elutriation experiments. The translocation of cyclin B1 to the nucleus was confirmed by Western analysis of cyclin levels in nuclear extracts of endomitotic cells. Nuclear cyclin B1 is detected in endomitotic HEL cells throughout the 7-day period of induction (unpublished observation).

DISCUSSION

Our data demonstrates that the mitosis-inducing cdk1/cyclin B1 protein kinase complex is differentially modulated during polyploidization. We hypothesized that for a cell to become polyploid, the cell must be altered such that an increased DNA content is retained within a single nucleus of that cell. This implies that biochemical control of mitosis should be modulated to both abrogate mitosis as well as dissociate the completion of S-phase from mitosis. We previously demonstrated that PMA induction in HEL cells results in a polyploid DNA content contained within a single nucleus (Long *et al.*, 1990). We now show that novel changes in the cdk1/cyclin B1 complex occur during the process of polyploidization. In endomitotic cells, the relative abundance of cdk1 is markedly reduced, and that of cyclin B1 is elevated. Consistent with the lack of the apoenzyme cdk1, endomitotic cells have little detectable cdk1-associated H1 histone kinase activity. The relative absence of cdk1 in these cells was corrected by the overexpression of this protein from a constitutive promoter. However, re-establishing cdk1 levels failed to either reconstitute H1-histone kinase activity or to inhibit endomitosis, due to the inability of cyclin B1 to physically associate with cdk1. A number of changes in endomitotic cells suggest a distinct role for cyclin B1 in the process of polyploidization. Cyclin B1 levels are increased as a result of both an increase in the abundance of cyclin B1 mRNA, as well as a prolongation of the half-life of the protein. Moreover, cyclin B1 is modified such that it cannot physically associate with cdk1. Cyclin B1 also translocates to the nucleus of endomitotic cells, and retains H1 histone kinase activity–even in cytoplasmic extracts exhaustively depleted of cdk1.

A disruption of the temporal dependence of mitosis on S-phase occurs in endomitotic HEL cells due to the loss of the cdk1/cyclin B1 protein kinase activity. This, in turn, is a result of a specific depletion of cdk1. This observation is consistent with previous data showing that depletion of cdk1 kinase activity or injection of anti-cdk1 antibodies blocks entry into mitosis, thus causing cell cycle arrest (Riabowol *et al.*, 1989; Fang and Newport, 1991; Th'ng, 1992), or that mutations in cdk1 (cdc2) disrupt the dependency of S-phase on the completion of the previous mitosis (Broek *et al.*, 1995). Our observations also confirm and extend that of Hayles *et al.* (1994), who demonstrated that a loss of the cdk1/cyclin B complex (i.e., $P34^{cdk1}/P56^{cdc13}$) in yeast results in multiple S-phases. This led these authors to define the G_1- and the G_2-phases of the cell cycle based on the absence or presence (respectively) of active cdk1/cyclin B complex. Our data confirm this observation in that the absence of the cdk1/cyclin B1 complex defines cellular conditions under which polyploidy is initiated. However, in contrast to Hayles *et al.*, our data show that, in human cells, it is a decrease in cdk1 protein and not cyclin B1, that aborts the formation of the kinase complex. Thus, one cannot conclude that the simple lack of functional cyclin B1 allows re-replication, as might be suggested from *Drosophila* embryo studies showing that cyclin B is absent during multiple rounds of S-phase (Lehner and O'Farrwll, 1990). Interestingly, recent work by Grafi and Larkins (1995) shows that endoreduplication leading to polyploidy in maize endosperm involves both an increase in the amount and activity of S-phase–related protein kinases, as well as active inhibition of MPF activity. We observe similar changes in endomitotic human cells (including S-phase kinases, see below), and show that the loss of MPF activity is related to both a reduction in cdk1 half-life, and an inability of cyclin B1 to complex with cdk1.

We reasoned that overexpression of cdk1 in endomitotic cells might reconstitute the formation of the cdk1/cyclin B1 protein kinase complex. However, we observed that cdk1 overexpression fails to prevent endomitosis. This failure to reconstitute kinase activity was found to be due to the inability of cdk1 to physically associate with cyclin B. The mechanism of this lack of physical association is most likely caused by a specific post-translational modification of cyclin B1. The nature of this modification is unknown, but it may occur within or near the "cyclin box." Cyclins interact

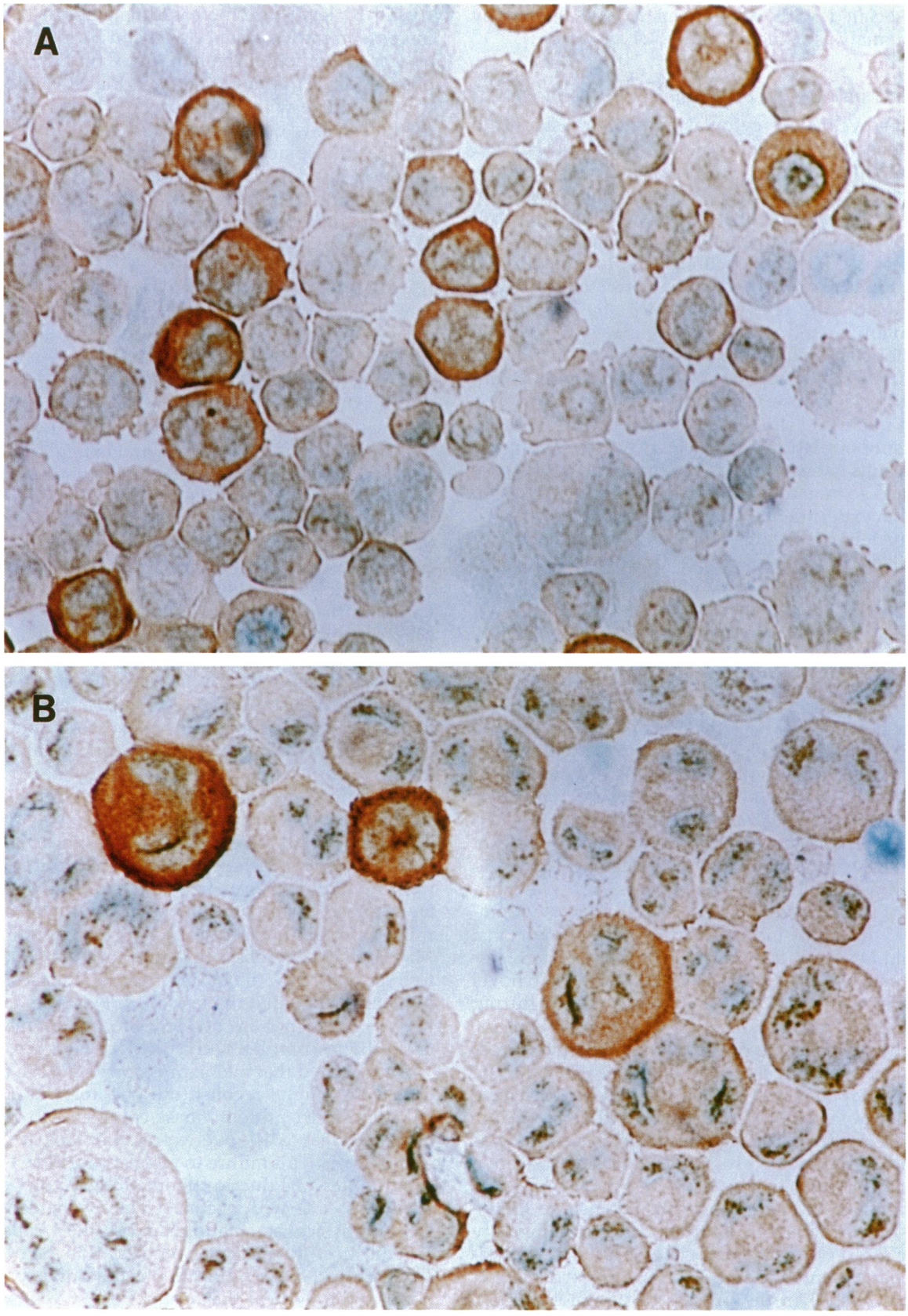

Figure 7.

with CDKs via two distinct regions of the cyclin molecule, and these "interaction domains" are necessary and sufficient for cyclin activation of the CDK (Lees and Harlow, 1993). Thus, we would predict that cyclin B1 is modified within this region to prevent its physical association with cdk1. Interestingly, cyclin B1 is also stabilized in endomitotic cells with its half-life being increased approximately threefold. Thus, cyclin-box or other modifications of cyclin B1 may result in the reduced degeneration of this protein. Alternatively, the cdk1/cyclin B1 complex may dissassemble due to the lack of specific proteins that regulate the association of cdk1 with cyclins, as has been shown for cdc37 in yeast cdc28/cln2 complexes (Gerber *et al.*, 1995), or by the absence of kinase-activating proteins as shown for mammalian cdk4/cyclin D complexes (Kato *et al.*, 1995).

A novel role for cyclin B1 in endomitosis was suggested by two observations. The first is that immunoprecipitates of cyclin B1 from endomitotic cells (which lack detectable cdk1) retain H1 histone kinase activity, even when these cytoplasmic extracts are taken through multiple rounds of cdk1 immune depletion. The second observation is that cyclin B1 is physically translocated to the nucleus in endomitotic cells. Western analysis of cyclin B1 immunoprecipitates from endomitotic cells demonstrates that cyclin B is not associated with PSTAIR-containing proteins (PSTAIR is the consensus amino acid sequence found in three members of the CDK gene family; cdk1-cdk3), nor is it associated with cdk4. However, distant members of the CDK family (cdk 5–7) are not detectable with the anti-PSTAIR antibody, so these CDKs cannot be excluded as candidate apoenzymes. Additionally, we have noted that, in immunoprecipitates of metabolically labeled cells, a single protein of ≈90–100 kDa is newly associated with cyclin B1 in endomitotic cells (Datta, unpublished observation). The nature of this protein is currently under investigation.

Consistent with a distinct role in endomitosis, we observed that cyclin B1 is specifically translocated to the nucleus of polyploid cells. This represents a unique cellular distribution for cyclin B1, as this protein is normally localized to the cytoplasm, specifically to microtubules, and abruptly translocates to the nucleus at the onset of mitosis (Pines and Hunter, 1991, 1994; Gallant and Nigg, 1992; Jackman *et al.*, 1995). Although the exact mechanism of cyclin B1 translocation is unknown, it is known that cyclin B contains an N-terminal cytoplasmic localization sequence (at amino acids 88–154) (Pines and Hunter, 1994). This region is a dominant regulator of cyclin B localization, suggesting that endomitotic cyclin B1 is either modified to cause this translocation or, alternatively, it may "piggy-back" with another protein (e.g., the endomitotic cyclin B1–associated apoenzyme) into the nucleus as has been suggested by Maridor *et al.*, 1993. The stabilization of cyclin B1 protein may also be important to the process of endomitosis. Structurally, the B cyclins contain a consensus sequence required for their destruction (the so-called destruction box) (Glotzer *et al.*, 1991). This destruction box is an ubiquitination site that marks the protein for subsequent degradation. Recent observations demonstrate that overexpression of truncated cyclin B results in mitotic arrest, as does expression of proteolytically stable intact cyclin B (via destruction box substitution mutations) (Luca *et al.*, 1991; Gallant and Nigg, 1992). Thus, the sustained presence of cyclin B causes mitotic arrest (but not polyploidy) in these cells. However, the mere presence of elevated cyclin B alone is not sufficient to cause polyploidy as none of the cells in the above reports contain polyploid nuclei.

We conclude that novel changes in cdk1/cyclin B1 complex formation occur during endomitosis. The process of mitosis is abrogated due to the lack of a functional M-phase promoting activity, thus resetting the cell to a "G_1-state" and allowing further (repetitive) S-phases. An important role for cyclin B1 in this process is strongly suggested by the multiple observations concerning this protein: increased mRNA, increased protein half-life, translocation of the protein to the nucleus, and the presence of a cyclin B1–associated histone kinase activity that is not caused by cdks 1–4. Although these changes argue persuasively for a distinct role for cyclin B1 in endomitosis, they do not, of course, show that these changes cause polyploidy. We have made a number of attempts to determine the role of cdk1 reduction, or cyclin B elevation, in the mechanism of endomitosis. For example, overexpression of cdk1 fails to reconstitute MPF, due to the inability of endomitotic cyclin B1 to physically associate with cdk1. Likewise, unpublished attempts at antisense oligodeoxynucleotide inhibition of cyclin B1 failed–perhaps due to the stabilization of (pre-existing) cyclin B1 in endomitotic cells. We also utilized lipofection of antibodies to cyclin B1 in an attempt to block polyploidy. Utilizing conditions that were carefully controlled for protein/lipid ratios, anti-cyclin B1 lipofection was seen to result in apoptosis of the anti-cyclin B1–treated cells, but not cells treated with an irrelevant antibody (Datta and Long, unpublished ob-

Figure 7 (facing page). Cellular localization of cyclin B1 in control and endomitotic cells. The translocation of cyclin B1 to the nucleus of endomitotic cells is demonstrated by immunocytochemical (peroxidase based) staining. (A) Control cells show a cytoplasmic cyclin B1 distribution in ~20% of the cells, a frequency consistent with the number of cells in G_2/M-phase (Figure 2). (B) Endomitotic cells, in contrast, all contain nuclear cyclin B1 as demonstrated by the granular brown reaction product clearly visible against the blue-green nuclear counterstain. As well, a subpopulation of large, endomitotic cells (of unknown significance) is seen that contains both cytoplasmic and nuclear cyclin B1 (the three darkely stained cells in the upper left to center of the field).

servations). Although programmed cell death is clearly an alternative fate for anti-cyclin B1-treated cells, its presence precludes a determination of the role of cyclin B1 in endomitosis. Finally, recent data (Datta and Long, unpublished observations) have shown that S-phase CDK complexes (i.e., cdk2/cyclin D_3 and cdk2/cyclin E) are differentially regulated during mitosis. Thus, as predicted, the acquisition of a polyploid nucleus involves a multi-step process affecting both S- and M-phase. Such complexities suggest that single or even double genetic manipulations would not demonstrate causal relationships. Nonetheless, we believe that the unique modulation of the cdk1/cyclin B1 complex plays an important role in endomitosis, as its absence ensures that the mitotic machinery is not assembled. Further, the elevated and sustained presence of cyclin B1 in the nucleus of endomitotic cells, together with its kinase activity, strongly suggests a novel function for this "mitotic" cyclin in endomitosis. Further characterization of these changes will provide important information regarding cell cycle control and the role of cyclin B1 in the process of polyploidization.

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