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Premature Expression of Cyclin B Sensitizes Human HT1080 Cells to Caffeine-Induced Premature Mitosis

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Abstract Eukaryotic cells do not normally initiate mitosis when DNA replication is blocked. This cell cycle checkpoint can be bypassed in some cells, however, by treatment with caffeine and certain other chemicals. Although S-phase arrested hamster cells undergo mitosis-specific events such as premature chromosome condensation (PCC) and nuclear envelope disassembly when exposed to caffeine, human cells show little response under the same conditions. To further investigate the molecular basis of this cell type specificity, a panel of hamster/human whole cell hybrids was created. The frequency of caffeine-induced PCC and the level of cyclin B-associated H1 kinase activity in the various hybrids were directly correlated with the extent of cyclin B synthesis during S-phase arrest. To determine whether expression of cyclin B alone could sensitize human cells to caffeine, cyclin B1 was transiently overexpressed in S-phase arrested HT1080 cells. The transfected cell population displayed a 5-fold increase in the frequency of caffeine-induced PCC when compared with normal HT1080 cells, roughly equivalent to the frequency of cells expressing exogenous epitope-tagged cyclin B1. In addition, immunofluorescent microscopy showed that individual cells overexpressing cyclin B1 during S phase arrest underwent PCC when exposed to caffeine. These results provide direct evidence that premature expression of cyclin B1 can make cells more vulnerable to chemically-induced uncoupling of mitosis from the completion of DNA replication.

Key words: cell cycle, cyclins, premature chromosome condensation, cyclin dependent kinases, cell cycle checkpoints

The initiation of mitosis in eukaryotic cells is controlled by maturation promoting factor (MPF) [for reviews see Norbury and Nurse, 1992; Coleman and Dunphy, 1994]. MPF consists of a 34 kDa serine/threonine protein kinase, the product of the *cdc2* gene, and a B-type cyclin regulatory subunit. At mitosis, MPF kinase activity rises dramatically, leading to phosphorylation of the nuclear lamins and numerous chromatin-associated proteins [Nigg, 1993]. The activity of $p34^{cdc2}$ is regulated by association with cyclin proteins and by phosphorylation. Upon binding with cyclin B, which is synthesized during late S/G_2 and localized in the cytoplasm [Pines and Hunter, 1989, 1994], $p34^{cdc2}$ is phosphorylated at multiple sites (Thr-14, Tyr-15, and Thr-161). The inhibitory phosphorylation at Tyr-15 is maintained by the protein kinase products of the *wee1* and *mik1* genes [Lundgren et al., 1991; Parker et al., 1992; McGowan and Russell, 1993] and removed by the tyrosine phosphatase cdc25C [Millar et al., 1991; Lee et al., 1992; Strausfeld et al., 1994]. The activating phosphorylation at Thr-161 is maintained by the cdc2 activating kinase (CAK) [Fesquet et al., 1993; Poon et al., 1993; Solomon et al., 1993; Wu et al., 1994]. Cyclin B degradation is required for cells to exit mitosis [Murray et al., 1989] and is associated with decreased kinase activity of MPF [Pines and Hunter, 1989].

Normally, cells arrested in S phase by DNA synthesis inhibitors such as hydroxyurea will not progress into mitosis [for review see Hartwell and Weinert, 1989]. This mitotic checkpoint can be bypassed by alterations of specific cellular proteins and by treatment of cells with certain chemicals. For example, mutation of Tyr-15 to Phe-15 in $p34^{cdc2}$ of *Schizosaccharomyces pombe* and combined mutations of Thr-14 to Ala-14 and Tyr-15 to Phe-15 in human $p34^{cdc2}$ allow mitosis to occur in the presence of DNA synthesis inhibitors [Gould and Nurse, 1989; Norbury et al., 1991]. Similarly, overexpression of cdc25

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in wee1 mutants of *S. pombe,* overexpression of the Drosophila cdc25 homologue *string* in *Xenopus* egg extracts, or the loss of mik1 and wee1 in *S. pombe* lead to premature mitosis in the presence of incompletely replicated DNA [Enoch and Nurse, 1990; Kumagai and Dunphy, 1991; Lundgren et al., 1991]. In addition, the nuclear protein complex RCC1-Ran negatively regulates mitotic onset [Nishimoto et al., 1978; Ren et al., 1993], and the loss of RCC1 function causes premature mitosis in the presence of DNA synthesis inhibitors [Nishitani et al., 1991; Dasso et al., 1992].

Chemical compounds such as caffeine [Schlegel and Pardee, 1986], the protein phosphatase inhibitor okadaic acid [Yamashita et al., 1990; *Steinmann et al.*, 1991], and the protein kinase inhibitors 2-aminopurine, 6-dimethylaminopurine, and staurosporine [Schlegel et al., 1990; Tam and Schlegel, 1992] have also been shown to induce premature mitosis in hamster cells arrested in S phase by DNA synthesis inhibitors. The mechanisms by which these chemicals bypass the mitotic checkpoint involves dephosphorylation of Tyr-15 on $p34^{cdc2}$ and activation of MPF [Yamashita et al., 1990; Steinmann et al., 1991; Tam and Schlegel, 1992]. Smythe and Newport have proposed that both caffeine and okadaic acid promote the dephosphorylation of $p34^{cdc2}$ by inhibiting wee1 and mik1-like kinases [Symthe and Newport, 1992].

Surprisingly, human cells are resistant to chemically induced premature mitosis [Steinmann et al., 1991; Schlegel and Craig, 1991]. It was observed that hamster cells synthesize cyclin B and form a cyclin B/$p34^{cdc2}$ complex during S-phase arrest while human cells do not [Steinmann et al., 1991]. Since cyclin B is required for mitosis, a molecular explanation for this species difference was suggested. To investigate this possibility further, we created a panel of hamster/human hybrids between Chinese hamster embryo fibroblasts (CHEF18) and human fibrosarcoma cells (HT1080) and found that the amount of cyclin B synthesis in S-phase arrested hybrids correlated with their ability to undergo caffeine-induced premature mitosis. To determine whether cyclin B expression alone was sufficient for this response, epitope-tagged human cyclin B1 was transiently overexpressed in HT1080 cells. When these cells were arrested in S phase and exposed to caffeine, they displayed an increased sensitivity to chemically induced premature mitosis.

MATERIALS AND METHODS

Cell Culture

Human fibrosarcoma cells (HT1080), Chinese hamster embryo fibroblasts (CHEF18), and all whole cell hybrids were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% iron-supplemented calf serum (HyClone, Logan, VT) and 4 mM glutamine in a water-saturated 10% CO_2/90% air atmosphere. New cultures were started from frozen stocks every 4–6 weeks. Cells were *Mycoplasma*-free based upon fluorescent staining with Hoechst 33258. S phase arrested cells were accumulated in 2.5 mM hydroxyurea for 5 h and collected by scraping. Mitotic cells were obtained by treating an asynchronous culture with nocodazole (0.1 μg/ml, Sigma Chemical Co., St. Louis, MO) for 8 h and then harvesting by gentle physical detachment [Tobey et al., 1967]. Caffeine-induced premature mitotic cells were accumulated by treating cells with 2.5 mM hydroxyurea for 5 h and then adding 5 mM caffeine along with 0.1 μg/ml nocodazole for an additional 8 h.

Cell Fusions

Fusions between human HT1080 cells (previously transfected with pSV2neo to confer resistance to G418, GIBCO/BRL Inc., Gaithersburg, MD) and hamster CHEF18 cells were performed as described previously [Davidson and Gerald, 1976]. Briefly, 1×10^6 CHEF18 cells were mixed with the same number of HT1080 cells and seeded into 25 cm^2 flasks. After overnight incubation, the nearly confluent cultures were rinsed once with phosphate-buffered saline (PBS), exposed for exactly 1 min to 2 ml of 48% polyethylene glycol (PEG 1000, Boehringer Mannheim, Indianapolis, IN) in pre-warmed (37°C) DMEM containing 10% serum and washed three times with PBS. After the addition of fresh growth medium, cells were incubated overnight. On the following day, cells were replated at one fifth the original density and grown in 500 μg/ml G418 to select against hamster parental cells and 2 μM ouabain (Sigma) to select against human parental cells. Individual resistant hybrid colonies were selected with cloning rings and propagated in DMEM supplemented with 500 μg/ml G418 and 2 μM ouabain. Identical treatment of human and hamster parental cells yielded no resistant clones.

Preparation of Chromosome Spreads

The percentage of cells undergoing premature mitosis was determined by chromosome spreads as described previously [Tam and Schlegel, 1992]. Briefly, cells were swelled for 10 min in 75 mM KCl, fixed for 10 min in methanol/acetic acid (3:1, vol./vol.), and dropped on wet slides before staining with Hoechst 33258 (1 μg/ml) for 7 min. Three hundred cells were examined by fluorescence microscopy at ×400 magnification to determine the fraction of cells having undergone premature mitosis. Nuclear envelope breakdown and chromatin pulverization were used as indicators of cells in S phase that were forced into premature mitosis [Johnson and Rao, 1970].

Immunoprecipitation

Cells were labeled with 100 μCi/ml ^{35}[S]-methionine/cysteine (ICN, Costa Mesa, CA; Tran ^{35}S-Label) for 3 h in methionine-deficient DMEM containing 10% dialyzed calf serum and then lysed for 30 min at 4°C in lysis buffer containing 1% Triton X-100, 5 mM EDTA, 20 mM Tris (pH 7.4), 0.1 M NaCl, 250 μM p-amidinophenol methanesulfonylfluoride (PMSF), 1 mM Na_3VO_4, 2 mM EGTA, 5 mM NaF, 12 mM 2-glycerol phosphate, and 1 mM ATP. An equal amount of trichloroacetic acid-precipitable radioactivity (1×10^7 cpm) from each extract was mixed with a polyclonal antiserum (1:100 dilution for 1.5 h at 4°C) raised against full length human cyclin B1 protein [Meikrantz et al., 1994]. Antibody-antigen complexes were precipitated with protein A sepharose slurry, added at one tenth of the extract volume, at 4°C for 1 h. The sepharose beads were washed 5 times in lysis buffer before the immunoprecipitated proteins were boiled in SDS Laemmli buffer [Laemmli, 1970], separated on a 10% SDS-polyacrylamide gel, and visualized by fluorography.

Histone H1 Kinase Assay

The histone H1 kinase assay was a modification of the procedures used by Yamashita et al. [1990]. Cells were washed twice with ice-cold PBS, lysed in lysis buffer (20 mM HEPES-NaOH (pH 7.5), 12 mM 2-glycerophosphate, 5 mM NaF, 1 mM ATP, 1 mM Na_3VO_4, 2 mM EGTA, 250 mM NaCl, 15 mM $MgCl_2$, 1% Triton X-100, 10 μg/ml each of aprotinin, leupeptin, and pepstatin and 0.5 mM PMSF), passed through a 25 gauge needle ten times, kept on ice for 30 min and clarified by centrifugation. Protein from the supernatant (50 μg as determined with the Pierce Micro BCA Protein Assay Reagent kit, Pierce, Rockford, IL) in a final volume of 200 μl was mixed for 1 h at 4°C with human cyclin B1 polyclonal antibody at 1:100 dilution. Antibody and associated proteins were affinity-precipitated by mixing with 100 μl of protein-A sepharose beads (Pharmacia Inc., Piscataway, NJ) at 4°C for 30 min. The sepharose beads were then washed twice in 500 μl lysis buffer and three times in 500 μl kinase buffer (20 mM HEPES-NaOH [pH 7.5], 15 mM EGTA and 20 mM $MgCl_2$). Histone H1 kinase activity was determined by incubation of the sepharose beads in 30 μl kinase buffer supplemented with 1 mM dithiothreitol, 0.01 μg/μl A-kinase inhibitory peptide (Sigma), 0.5 μg/μl histone H1 (Boehringer Mannheim), and 50 μM ATP (5,500 c.p.m./pmol) for 20 min at 30°C. The reaction was stopped by adding 30 μl of 2 × Laemmli sample buffer and boiling for 10 min. Proteins were separated in a 12% SDS-PAGE mini-gel, fixed in 25% trichloroacetic acid, stained with Coomassie brilliant blue R and relative kinase activity assessed by autoradiography.

Transient Expression of Cyclin B1

HT1080 cells were plated in 60-mm dishes, grown to 50% confluence and transfected by lipofection (GIBCO/BRL), using the protocol recommended by the manufacturer. The cDNA encoding human cyclin B1 [Pines and Hunter, 1989] was tagged with a myc epitope at the N-terminus and fused with the lamin A 5′ untranslated sequence. Restriction sites were added by polymerase chain reaction before cloning into the mammalian pCMV1 expression vector [Heald et al., 1993]. For each transfection, 3 μg of human cyclin B1 plasmid DNA (pCMV1CYCB1; a gift of Frank McKeon, Harvard Medical School) was diluted to 30 μl in water, filtered through a 0.22 μm membrane, combined with 30 μl Lipofectin reagent (1 μg/μl, GIBCO/BRL Inc.) and incubated for 15 min at room temperature. Cells were washed twice with 5 ml sterile PBS and overlayed with 3 ml Opti-MEM I reduced serum medium (GIBCO/BRL Inc.). The Lipofectin reagent-DNA complex was then added to the cells dropwise and swirled gently to mix. Cells were incubated for 12 h before returning to normal DMEM. After an additional 18 h incubation, cells were arrested in S phase with 2.5 mM hydroxyurea for 5 h before *0.1 μg/ml noco-*

dazole with or without 5 mM caffeine was added for an additional 18 h. Chromosome spreads were prepared as above and immunofluorescent microscopy was performed as described below to determine the frequency of cells expressing exogenous cyclin B1 and the percentage of cells undergoing premature mitosis.

Immunofluorescence Microscopy

HT1080 cells plated on 12 mm glass coverslips were transiently transfected with cyclin B1 as above and fixed for 10 min in 2% formaldehyde in PBS. After fixation, cells were rinsed three times with 0.1% NP40 in PBS (NP40-PBS) and incubated for 1 h at room temperature (1:20 dilution in NP40-PBS) with mouse monoclonal anti-myc IgG1 antibody (9E10) [Evan et al., 1985]. After rinsing 3 times with NP40-PBS, cells were incubated for 1 h with rhodamine-conjugated goat anti-mouse secondary antibody (Boehringer Mannheim, diluted 1:100 in NP40-PBS). After 3 more rinses with NP40-PBS, cellular DNA was stained with 10 μg/ml Hoechst 33258 (Sigma) for 1 min. Following 3 additional washes with NP40-PBS, coverslips were mounted with cells facing down on a drop of 0.1M N-propylgallate in 80% glycerol and sealed with nail polish. Micrographs were taken with a Zeiss (Thornwood, NY) fluorescent microscope using a ×100 oil objective and Kodak (Rochester, NY) Ektachrome P800/1600 film.

RESULTS

Premature Mitosis in Hamster/Human Hybrids

Caffeine has been shown to induce S-phase PCC in hamster cells but not in human cells [Schlegel and Pardee, 1986; Steinmann et al., 1991]. To examine the molecular differences that contribute to this species specific response, we created a panel of ten whole cell hybrids by fusing hamster CHEF18 and human HT1080 cells. These hybrids, resistant to both G418 and ouabain, all have chromosome numbers significantly higher than either parental cell line, further confirming successful cell fusion (data not shown). The parental lines and whole cell fusion (WCF) clones were arrested in S phase, exposed to caffeine, and assessed for sensitivity to caffeine-induced PCC by chromosome analysis (Fig. 1). HT1080 cells showed less than 1% PCC, while CHEF18 cells exhibited 55% PCC. The ten hybrids differed greatly in response, covering the entire range between the two parental lines. Three of the hybrids (WCF 2, 7, and 8) were

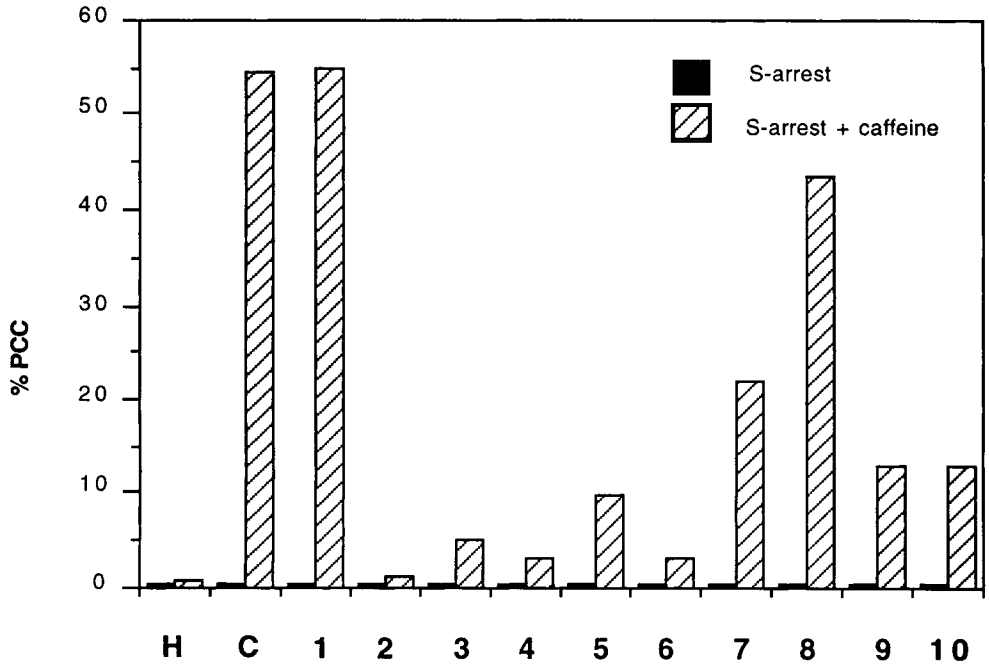

Fig. 1. Caffeine-induced PCC in parental cells and whole cell fusion hybrids. HT1080 (H), CHEF18 (C), and hamster/human whole cell hybrids (1–10) were arrested in S phase with hydroxyurea (2.5 mM) for 13 h. Premature mitotic cells were accumulated in the presence of caffeine (5 mM) and nocodazole (0.1 μg/ml) during the final 8 h of hydroxyurea treatment. Chromosome spreads were prepared as described in Materials and Methods and 300 cells of each cell type were examined for premature mitosis.

examined further because they displayed low (2% PCC), medium (20% PCC), and high (45% PCC) responses to caffeine, respectively.

The cytoplasmic and nuclear morphology of cells undergoing PCC is shown in Figure 2. Cells arrested in S phase with hydroxyurea retained an interphase morphology (Fig. 2A). After treatment with hydroxyurea and caffeine, however, the parental CHEF18 cells "rounded up" and resembled cells undergoing mitosis. Chromosome spreads showed chromosome pulverization and nuclear envelope breakdown, indicative of S-phase arrested cells forced into premature mitosis [Johnson and Rao, 1970; Schlegel and Pardee, 1986]. The parental HT1080 cells did not undergo these changes. The responses of WCF 2, 7, and 8 are shown in Figure 2B. The pulverized appearance of the chromatin, which is shown for WCF 8, was similar in all hybrid cells undergoing PCC and was indistinguishable from that seen in CHEF18 parental cells. These hybrids were then used to investigate the relationship between the sensitivity to caffeine-induced PCC and the premature expression and kinase activity of the mitotic regulatory proteins cyclin B and $p34^{cdc2}$.

Responsiveness to Caffeine-Induced PCC Is Correlated With Cyclin B Synthesis During S Phase Arrest

We had determined earlier that hamster cells synthesize cyclin B during S phase arrest while human cells do not [Steinmann et al., 1991]. As an initial step toward causally linking the timing of cyclin B synthesis with sensitivity to chemically induced PCC, we examined the synthesis of cyclin B during S phase arrest in hamster and human parental cells and in hybrids that displayed various degrees of sensitivity to caffeine-induced PCC (WCF 2, 7, and 8). Following arrest in S phase with hydroxyurea, cells were labelled with ^{35}[S]-methionine/cysteine and the extent of cyclin B synthesis was determined by immunoprecipitation with cyclin B antibody (Fig. 3). Little or no labelled cyclin B was detectable in S-phase arrested human HT1080 cells, while cyclin B had begun to accumulate in similarly treated hamster CHEF18 cells. As expected, hamster and human parental cells arrested in mitosis with nocodazole synthesized significantly more cyclin B than the corresponding cells arrested in S phase. Consistent with the hypothesis that the accumulation of cyclin B during S phase arrest represents the critical molecular event that sensitizes cells to chemically-induced PCC, the level of cyclin B in S-phase arrested hamster/human hybrids was directly related to the responsiveness of these hybrids to caffeine-induced PCC. WCF 2, which displayed very low levels of PCC, contained essentially undetectable levels of cyclin B. WCF 7 and 8, which displayed intermediate and high levels of PCC, respectively, contained intermediate and high levels of cyclin B. No significant differences were found in the level of expression of $p34^{cdc2}$, the catalytic partner of cyclin B, in the parental and hybrid lines (data not shown).

The cyclin B synthesized in the hybrids is derived from hamster and not human chromosomes because the cyclin B antibody used in these experiments is able to immunoprecipitate both human and hamster cyclin B but only recognizes human cyclin B on immunoblots. Immunoblots of S phase arrested hybrids did not reveal any human cyclin B (data not shown). The low level of cyclin B synthesis in WCF 2 could be due to either the loss of hamster chromosomes containing cyclin B or to suppression of cyclin B expression. Interestingly, both WCF 7 and 8 displayed higher levels of cyclin B during S phase arrest than did the CHEF18 parental cell line, even though CHEF 18 cells were more sensitive to caffeine-induced PCC than either of these hybrids. This apparent discrepancy may result from the more difficult task of condensing at least twice as much chromatin in the hybrids as in the hamster parental cells. A similar phenomenon was reported by Rao [1982] when mitotic cells were fused with increasing numbers of interphase cells. As the DNA content of the fused cells increased, the ability of mitotic cells to drive interphase cells into PCC decreased dramatically. The possibility also exists that hamster cyclin B is not as effective at inducing mitosis or activating $p34^{cdc2}$ in hamster/human hybrids as it is in hamster parental cells.

Caffeine-Induced Histone H1 Kinase Activity Is Directly Related to the Level of PCC in the Hybrids

To assess the functional consequences of premature synthesis of cyclin B during S phase arrest, we determined the cyclin B-associated histone H1 kinase activities of parental and hybrid cells during mitosis and during S phase arrest with and without caffeine treatment (Fig. 4). The kinase activities of all parental and hybrid lines was high in mitotic cells and very low

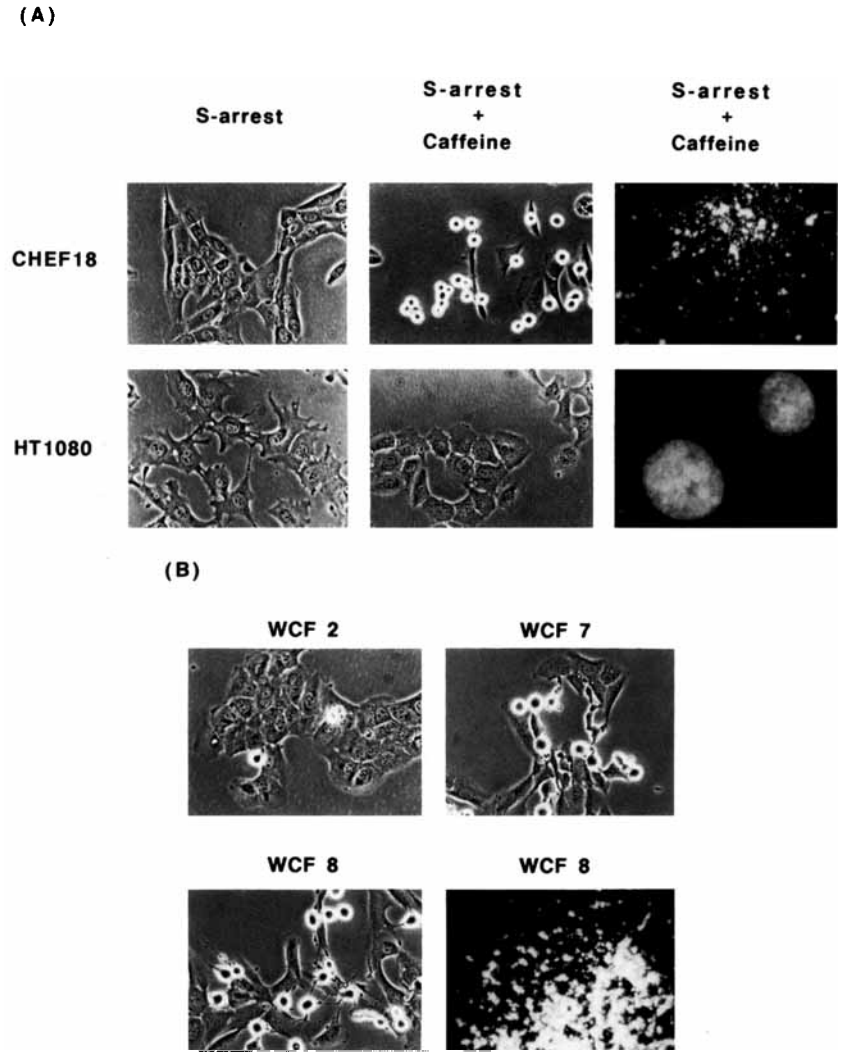

Fig. 2. Changes in cell and chromatin morphology during caffeine-induced premature mitosis. CHEF18, HT1080, and hamster/human whole cell hybrids (WCF) were arrested in S phase with hydroxyurea (2.5 mM) for 13 h and either remained untreated or were exposed to caffeine (5 mM) and nocodazole (0.1 μg/ml) during the final 8 h. **A:** Left and middle panels are phase contrast micrographs (×200) of CHEF18 and HT1080 cells. After caffeine treatment, CHEF18 cells, but not HT1080 cells, rounded up and became refractile. Right panels: chromosome preparations stained with Hoechst 33258 (×1,000) show chromosome pulverization and nuclear envelope breakdown in CHEF18 cells but not in HT1080 cells. **B:** Phase contrast micrographs (×200) of S-phase arrested WCF hybrids undergoing differing degrees of PCC following exposure to caffeine for 8 h. Bottom right panel: chromosome preparation (×1,000) stained with Hoechst 33258 showing WCF 8 cells undergoing caffeine-induced PCC.

in S-phase arrested cells, consistent with the earlier finding that cyclin B/p34^{cdc2} complexes that are formed in hamster cells during S phase arrest are normally inactive [Steinmann et al., 1991]. When arrested cells were exposed to caffeine, however, those cells that expressed cyclin B during S phase arrest (CHEF18, WCF 7 and 8) showed a dramatic increase in cyclin B-associated kinase activity, with the activity in CHEF18 cells approaching that seen during normal mitosis. HT1080 and WCF 2 cells displayed little or no increase in kinase activity following caffeine treatment, consistent with their lack of cyclin B while arrested in S phase. There was a strong correlation between the extent of PCC and the level of kinase activity, with kinase activity and frequency of caffeine-induced PCC both increasing in the order of WCF 2, WCF 7, WCF 8, CHEF18. This direct relationship was not necessarily expected. In the lower eukaryote

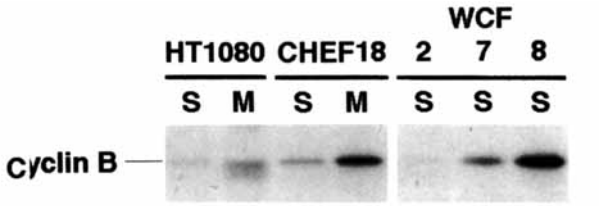

Fig. 3. Cyclin B synthesis in parental and hamster/human hybrids during S and M phases. Cyclin B was immunoprecipitated from whole cell extracts of parental cells and whole cell fusion (WCF) hybrids that were arrested either in S phase for 5 h with 2.5 mM hydroxyurea (S) or in mitosis for 8 h with 0.1 μg/ml nocodazole (M) before labelling with ^{35}[S]-methionine/cysteine (100 μCi/ml) for an additional 3 h. S phase cells were then collected by scraping and M phase cells were harvested by gentle physical detachment. Immunoprecipitations were performed using equal amounts of trichloroacetic acid-precipitable radioactivity (10^7 cpm). Immunoprecipitated proteins were separated in 12% polyacrylamide gels and detected by fluorography.

Saccharomyces cerevisiae, for example, arrest in S phase is accompanied by increased H1 kinase activity, and induction of kinase activity is not always sufficient to initiate mitosis [Sorger and Murray, 1992; Amon et al., 1992; Stueland et al., 1993].

As one might anticipate, caffeine-induced kinase activity during S phase arrest was a somewhat better predictor of sensitivity to caffeine-induced PCC than was the level of cyclin B synthesis under the same conditions. It is not clear why CHEF18 cells had higher caffeine-induced kinase activity than did WCF 7 and WCF 8 even though the latter cell lines displayed a higher synthesis of cyclin B during S phase arrest (see Fig. 3). It is possible that hamster cyclin B does not act as effectively in human cells as in hamster cells or that human cells express to a greater extent an inhibitory factor or factors that can partially suppress cyclin B/p34^{cdc2} kinase activity.

Transient Expression of Human Cyclin B1 Sensitizes HT1080 Cells to Caffeine-Induced PCC

While the above experiments using hamster/human hybrids provided results consistent with the hypothesis that the timing of cyclin B synthesis is an important contributor to the species-specific sensitivity to caffeine-induced PCC, it was not known whether cyclin B was the only component that was missing from human cells to account for this difference in response. To address this question, we transiently overexpressed cyclin B1 in S-phase arrested HT1080 cells to determine whether cyclin B1 expression alone could sensitize these cells to caffeine-induced PCC. The expression plasmid used, pCMV1CYCB1 [Heald et al., 1993], contained cyclin B1 tagged with a *myc* epitope at the N-terminus, and expression was driven by the cytomegalovirus promoter. Attempts to generate stable HT1080 lines that constitutively overexpressed cyclin B1 were unsuccessful, likely due to cytotoxicity resulting from the inability of cells to exit mitosis [Murray et al., 1989].

When HT1080 cells were transfected with pCMV1CYCB1, their ability to undergo caffeine-induced PCC was more than five-fold greater than both parental HT1080 cells and HT1080 cells transfected with control plasmid lacking the myc-tagged cyclin B1 gene (Fig. 5). Since the frequency of PCC in HT1080 cells transfected with cyclin B1 was only 2–3% of the total cell population, it was important to establish that the cells undergoing PCC were in fact those cells expressing the transfected cyclin B1. For these

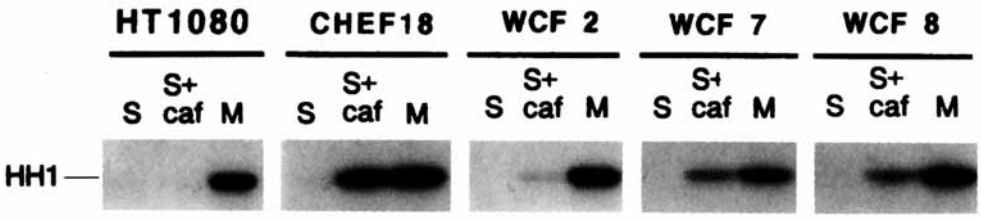

Fig. 4. Histone H1 kinase activation by caffeine. Cells were either arrested in S phase with hydroxyurea (2.5 mM) for 13 h (S), arrested in S phase as above and exposed to caffeine (5 mM) and nocodazole (0.1 μg/ml) during the final 8 h of hydroxyurea treatment to accumulate cells undergoing PCC (S + caf), or arrested in mitosis with nocodazole (0.1 μg/ml) for 8 hours (M). S phase and PCC cells were then collected by scraping; M phase cells were harvested by gentle physical detachment. Fifty micrograms of whole cell protein extract from each treatment group was assayed for cyclin B-associated histone H1 kinase activity. The phosphorylated histone H1 substrate (HH1) was detected by autoradiography after separation in polyacrylamide gels, as described in Materials and Methods.

experiments, transfected cells were arrested in S phase and either remained untreated or were exposed to caffeine as before. Following fixation, cells expressing transfected cyclin B1 were identified with a monoclonal antibody against the *myc* epitope tag and a rhodamine-conjugated secondary antibody. The condensation state of the chromatin was determined by co-staining with Hoechst 33258. Figure 6a shows the expected cytoplasmic localization of *myc*-tagged cyclin B1 during S phase arrest. The nuclei retained a normal interphase morphology (Fig. 6c; same field of cells as Fig. 6a), and chromosome preparations showed no indication of PCC or nuclear envelope breakdown (Fig. 6e). Upon treatment with caffeine, cyclin B1 immunostaining was present throughout the cells (Fig. 6b), cells expressing the *myc*-tagged cyclin B1 underwent chromatin condensation (Fig. 6d; same field of cells as Fig. 6b), and extensive chromatin pulverization and nuclear envelope breakdown were evident in chromosome preparations (Fig. 6f). As shown in Figure 6b and d, cells that were negative for cyclin B1 expression did not undergo chromatin condensation or nuclear envelope breakdown. The 2–3% frequency of PCC in the entire transfected cell population is essentially equivalent to the percentage of transfected cells that express the *myc*-tagged cyclin B1. These results indicate that the cells undergoing PCC in the transfected cell population are in fact the same cells that are expressing transfected cyclin B1, and demonstrate that expression of cyclin B1, by itself, is sufficient to sensitize HT1080 cells to chemically induced PCC. These findings also confirm earlier studies in hamster cells showing that cyclin B expression alone during S phase arrest was not sufficient to induce premature mitosis without additional chemical or enzymatic activation [Steinmann et al., 1991; Heald et al., 1993].

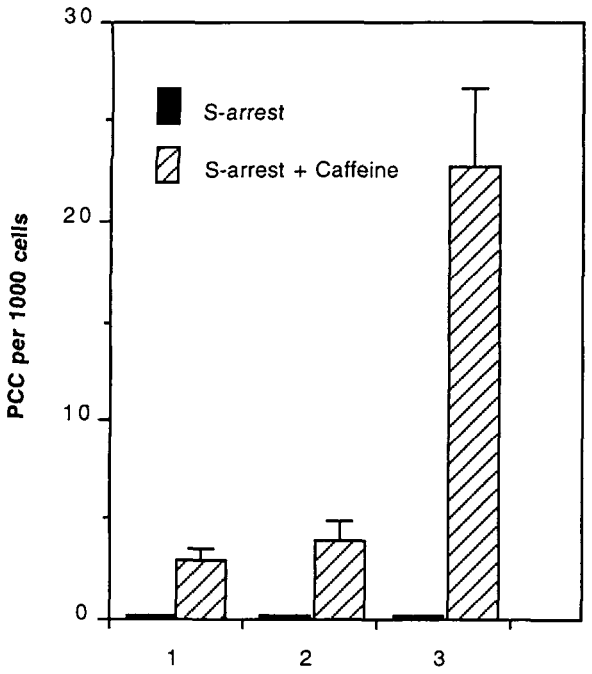

Fig. 5. Frequency of PCC in HT1080 cells transiently transfected with cyclin B1. HT1080 cells remained untransfected (1) or were transiently transfected with either pCMV1 control plasmid (2) or pCMV1CYCB1 (3). Thirty hours later, cells were arrested in S phase with 2.5 mM hydroxyurea for 13 h, with or without the addition of nocodazole (0.1 μg/ml) and caffeine (5 mM) during the final 8 h. Chromosome spreads were prepared as described in Materials and Methods and the frequency of cells exhibiting premature mitosis was determined. Three independent experiments were conducted for each treatment group and the error bars represent the standard error of the mean. One thousand cells were examined for each treatment group in each experiment.

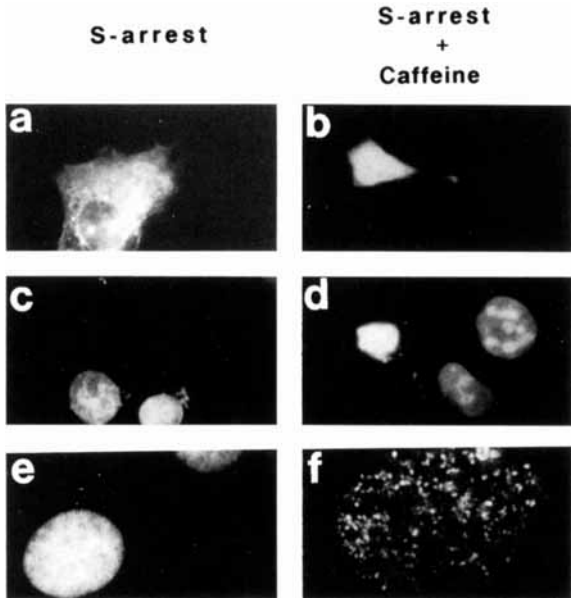

Fig. 6. Caffeine-induced PCC in individual cells overexpressing cyclin B1. HT1080 cells transiently transfected with pCMV1CYCB1 were arrested in S phase with 2.5 mM hydroxyurea for 13 h, either without **(a,c,e)** or with **(b,d, f)** the addition of caffeine (5 mM) and nocodazole (0.1 μg/ml) during the final 8 h. After fixation, *myc*-tagged cyclin B1 was visualized with anti-myc polyclonal antibody followed by secondary antibody conjugated to rhodamine. DNA was stained with the fluorochrome Hoechst 33258. Expression of *myc*-tagged cyclin B1 is shown in a and b, while the same cells stained with Hoechst 33258 are shown in c and d. Chromosome spreads of the two treatment groups, stained with Hoechst 33258, are shown in e and f.

DISCUSSION

Human cells, unlike hamster cells, exhibit little or no chemically induced premature mitosis when DNA replication is blocked [Steinmann et al., 1991]. This insensitivity spares human cells the cytogenetic damage and nuclear fragmentation that occurs when mitosis is initiated prior to the completion of DNA replication. In a related finding, Schimke and colleagues have found that hamster cells, but not human cells, undergo aberrant mitotic events and cell death when released from S phase arrest [Kung et al., 1990; Schimke et al., 1991]. It appears, therefore, that the normal progression of cell cycle events is more easily disrupted in hamster cells than in human cells. In another cell cycle pathway, inactivation of retinoblastoma protein function by viral proteins is correlated with the absence of cyclin D1/cdk4 complexes [Tam et al., 1994]. These complexes appear to be unaffected in mouse cells transformed by similar viral agents [Bates et al., 1994], perhaps making rodent cells more likely to lose early cell cycle checkpoints as well.

The species-specific sensitivity to chemically induced premature mitosis has been proposed to be due to differences in the timing of cyclin B synthesis. While hamster cells accumulate cyclin B and form $p34^{cdc2}$/cyclin B complexes during S phase arrest [Nishitani et al., 1991; Steinmann et al., 1991], human cells do not [Steinmann et al., 1991]. Consistent with this hypothesis is the finding that BHK hamster cells are insensitive to caffeine-induced premature mitosis when they are arrested in G1 [Schlegel and Pardee, 1986], a stage containing little cyclin B. When human and hamster cells are arrested in G2 by DNA damage, a period of cyclin B accumulation [Pines and Hunter, 1989], both cell types initiate mitosis prematurely when exposed to caffeine or several other chemicals [Steinmann et al., 1991]. In addition, simultaneous overexpression of cdc2 and cdc13 (cyclin B) in the yeast *Schizosaccharomyces pombe* causes cells arrested in G1 to enter mitosis [Hayles et al., 1994]. Results from the present study now indicate that expression of cyclin B alone during S-phase arrest sensitizes human HT1080 cells to chemically induced premature mitosis. However, premature expression of cyclin B is not sufficient for this response without combined chemical treatment.

We have also transiently overexpressed human cyclin B1 in another human cell line, HeLa cells, and found little or no increase in caffeine-induced PCC despite higher transfection efficiency in HeLa cells when compared with HT1080 cells (data not shown). This result implies that the mere formation of a pre-MPF complex is not sufficient to sensitize all cell types to chemically induced PCC. Related findings in yeast and certain filamentous fungi have shown that $p34^{cdc2}$/cyclin B is not the only mitotic factor required for mitosis. In *Aspergillus nidulans,* the protein kinase NimA works cooperatively with $p34^{cdc2}$ to trigger mitosis, and lack of NimA activity suppresses mitotic onset even in the presence of activated MPF [Osmani et al., 1991]. In the budding yeast *Saccharomyces cerevisiae,* cells arrested in S and G2 do not initiate mitosis despite the presence of activated MPF, indicating additional regulatory pathways governing mitotic onset in these cells [Sorger and Murray, 1992; Amon et al., 1992; Stueland et al., 1993]. HT1080 and HeLa cells, therefore, may differ in certain components of the mitotic regulatory pathways. Alternatively, the subcellular localization of the MPF complex and its regulators, cdc25 and wee1, might contribute to cell type differences in mitotic control [Heald et al., 1993]. For example, cdc25C was found localized to the nucleus of HeLa cells [Millar et al., 1991], while in hamster tsBN2 cells, which undergo PCC in response to either caffeine or loss of RCC1 protein at the restrictive temperature, cdc25C was found in the cytoplasm [Seki et al., 1992]. The presence of MPF-activating cdc25C in the cytoplasm of hamster cells, the same location as that for pre-MPF, may increase the sensitivity of these cells to chemically induced PCC.

The detailed mechanisms by which chemicals bypass mitotic checkpoints and induce premature mitosis are not fully understood. To date, all studies have shown that premature mitosis is accompanied by loss of the inhibitory phosphorylation on tyrosine-15 of $p34^{cdc2}$ and by activation of the kinase [e.g. Yamashita et al., 1990; Steinmann et al., 1991]. Smythe and Newport [1992] have proposed that caffeine and okadaic acid act to reduce the tyrosine phosphorylation state of $p34^{cdc2}$ by suppressing the $p34^{cdc2}$ tyrosine kinase(s). In *Xenopus* extracts, addition of okadaic acid leads to the accumulation of hyperphosphorylated and activated forms of cdc25

and the generation of MPF activity [Kumagai and Dunphy, 1992]. Microinjection of anti-cdc25C antibodies into hamster BHK cells partly inhibited both normal mitosis and caffeine-induced PCC [Seki et al., 1992]. However, little or no change in the phosphorylation state of cdc25C was observed when BHK cells underwent PCC in response to caffeine or staurosporine (Chang and Schlegel, unpublished results). Clearly, there remains much to be learned about the mechanisms by which chemicals override normal mitotic checkpoints, and this may require the identification of as yet undiscovered regulatory components.

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