Caffeine Overcomes a Restriction Point Associated with DNA Replication, but Does Not Accelerate Mitosis

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Abstract. Mitotic chromosome condensation is normally dependent on the previous completion of replication. Caffeine spectacularly deranges cell cycle controls after DNA polymerase inhibition or DNA damage; it induces the condensation, in cells that have not completed replication, of fragmented nuclear structures, analogous to the S-phase prematurely condensed chromosomes seen when replicating cells are fused with mitotic cells. Caffeine has been reported to induce S-phase condensation in cells where replication is arrested, by accelerating cell cycle progression as well as by uncoupling it from replication; for, in BHK or CHO hamster cells arrested in early S-phase and given caffeine, condensed chromosomes appear well before the normal time at which mitosis occurs in cells released from arrest. However, we have found that this apparent acceleration depends on the technique of synchrony and cell line employed. In other cells, and in synchronized hamster cells where the cycle has not been subjected to prolonged continual arrest, condensation in replication-arrested cells given caffeine occurs at the same time as normal mitosis in parallel populations where replication is allowed to proceed. This caffeine-induced condensation is therefore "premature" with respect to the chromatin structure of the S-phase nucleus, but not with respect to the timing of the normal cycle. Caffeine in replication-arrested cells thus overcomes the restriction on the formation of mitotic condensing factors that is normally imposed during DNA replication, but does not accelerate the timing of condensation unless cycle controls have previously been disturbed by synchronization procedures.

The onset of mitotic chromosome condensation is controlled by a cascade of events, fundamentally similar in yeast and in larger eukaryotes, that involves the accumulation of cyclin proteins, their association with the *cdc2* gene product, and changes in the phosphorylation state of the complex (for review, see reference 13). Initiation of this cascade, with subsequent chromosomal condensation, is normally dependent on the completion of earlier stages in the cell cycle. In the presence of inhibitors of DNA synthesis, cells with unreplicated genomes do not progress towards mitosis; and cells in which replication is retarded by DNA damage enter mitosis only after a delay. However, cells in earlier stages of interphase can respond adequately to the presence of condensation inducing factors, for interphase cells undergo premature chromosome condensation when fused with mitotic cells (8). In S-phase cells, this produces characteristic heterogeneous chromatin structures, with many fragmented regions of intense condensation separated by diffuser material (12).

Caffeine can override the delay in cycle progression that normally occurs after DNA damage (2, 11, 26). Recently Schlegel and Pardee have shown that, in BHK-21 Syrian hamster cells arrested at the start of S-phase, caffeine can similarly override the cycle delay and induce mitotic condensation even though the genome remains incompletely replicated (23). The resulting condensed nuclear structures are equivalent to the S-phase prematurely condensed chromosomes obtained by cell fusion. In BHK-21 hamster cells synchronized in early S-phase (by isoleucine depletion, which arrests cells in G_1, followed by prolonged hydroxyurea blockade, which prevents progression through S-phase), and treated with caffeine, S-phase condensation was found to begin within a few hours, long before such a synchronized population would enter mitosis if released from hydroxyurea (23–25). Similar results have been reported for CHO hamster cells after prolonged hydroxyurea arrest (1). It has therefore been supposed that caffeine-induced S-phase condensation, like the condensation induced by fusion with mitotic cells, is premature. Caffeine appears both to uncouple the mitotic condensation cycle from previous stages of cell cycle control, and to accelerate the timing of the mitotic cycle. An analogous situation occurs in the *tsBN2* mutants of BHK-21, which at the nonpermissive temperature moves rapidly into premature condensation (15). The mechanism for this con-

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densation, like the mechanism of caffeine's action, is uncertain though a human gene that restores the *tsBN2* defect has been cloned (16).

We have found that caffeine can similarly induce S-phase condensed chromosomes in mammalian cells where replication is delayed by DNA damage (14). However, with the SV40-transformed Indian muntjac cells we used, condensation was not premature. With a mitotically synchronized cell population UV-irradiated in G_1, the onset of caffeine-induced S-phase condensation coincided with the onset of normal mitosis in a control synchronized population. This failure of caffeine to accelerate chromosome condensation is apparently discordant with the results of Schlegel and Pardee and of Brinkley et al. (1, 23); however, the cell lines, methods of synchrony and agents of replication arrest were different. We have therefore investigated further the kinetics of induction of S-phase condensed chromosomes by caffeine.

Materials and Methods

Cell Culture, Synchrony, and Irradiation

CHO, BHK-21, and SVM cells were normally grown as monolayer cultures at 37°C in MEM supplemented with 10% FCS and antibiotics.

For synchrony at the start of S-phase, cells were subcultured at 10^5/30-mm dish, and grown for 24 h; they were then washed two times with PBS and given isoleucine-free F12 medium supplemented with 10% dialyzed FCS for 30 h; and then given normal medium containing 2 mM hydroxyurea for 18 h.

For synchrony in mitosis, proliferating cultures were treated for 4 h with 0.05 μgm/ml colcemid to accumulate mitotic cells, which were shaken off, washed, and plated out at 10^5/30-mm dish.

For synchrony in G_1, cells were either treated with isoleucine-free medium as above, without further hydroxyurea treatment; or they were subcultured and grown for 48 h in medium containing 5 mM sodium butyrate (6).

Cells were UV-irradiated with a mercury lamp emitting predominantly at 254 nm, as monolayer cultures with the medium removed.

Scoring of S-phase and Mitotically Condensed Figures

After synchrony, cell populations were either released into normal growth medium and allowed to progress through the cycle, or given fresh medium containing 2 mM caffeine and 2 mM hydroxyurea. Some samples were irradiated and given caffeine, or given caffeine and hydroxyurea, after release. In some cases, cells progressing through the cycle were accumulated in mitosis by treatment with 0.05 μgm/ml colcemid. At intervals, cells were trypsinized, hypotonically swollen, fixed, spread, stained with crystal violet, and scored for frequencies of S-phase condensed figures or mitotic chromosome figures as previously described (8).

BrdU Labeling and Flow Cytometry

Synchronized or unsynchronized populations of cells were labeled for 60 min with 10 μM bromodeoxyuridine, fixed, and stained with anti-BrdU mouse antibody and FITC-labeled goat anti-mouse antibody by the procedure of Dolbeare et al. (5), and treated with propidium iodide to stain DNA. Samples were diluted to $2\text{-}5 \times 10^5$ cells/ml, and the fluorescence patterns of samples of 10,000 cells were analyzed by flow cytometry using the MRC dual laser flow cytometer (Cambridge Instruments, Inc., Monsey, NY), which has been previously described (27, 28). The Innova 70 argon ion laser (Coherent Inc., Palo Alto, CA) was tuned to the 488-nm line at a light power of 200 mW, which elicited green fluorescence proportional to BrdU content and red fluorescence proportional to DNA content. Forward and 90° light scatter were also analyzed and the data were collected list-mode on a 640 Mbyte Maxtor disk via dedicated LSI-23 and time-sharing PDP 11/40 computers. The data were processed using a VAX 8600 (Digital Equipment Corporation) to produce a four-dimensional cross-correlated data file (29). Debris and clumps were gated out using the light scatter signals and pulse shape analysis (30) and the green (BrdU) versus red (DNA) data were then displayed as contour maps.

Results

Kinetics of S-phase Condensation

In early S-phase populations of BHK-21 or CHO cells obtained by the isoleucine depletion/hydroxyurea blockade method, and further treated with caffeine and hydroxyurea, S-phase condensed chromosomes appear rapidly, well before normal mitosis, in accordance with previous reports (1, 23–25) (Fig. 1, *a* and *b*). This caffeine-induced condensation is, as previously reported (24), transient and reversible; S-phase condensed cells disappear from the population before the time of normal mitosis. By contrast, SVM cells synchronized by this method do not form S-phase condensed chromosomes until 8–10 h after the addition of caffeine, which corresponds to the time of onset of normal mitosis in control cells released from the hydroxyurea blockade (Fig. 1 *c*).

Synchrony by shake-off in mitosis, after brief accumulation with colcemid, involves a less prolonged disturbance of the cell cycle. In SVM or CHO cells so synchronized, either hydroxyurea plus caffeine or UV plus caffeine, when given late in G_1, produces S-phase condensation only after a substantial delay, at the time of normal mitosis (Fig. 2). Treatment of SVM or CHO in late G_1 with caffeine alone does not significantly affect subsequent entry into mitosis (data not shown). In SVM, UV plus caffeine produces more S-phase condensation than in CHO, presumably because DNA synthesis in CHO cells is only temporarily suppressed by irradiation, whereas the suppression in SVM is severe and lasting (18). Again, this condensation of the unreplicated genome in SVM or CHO is transient. Attempts at shake-off of mitotic-arrested BHK-21 cells have not produced well-synchronized populations; this technique cannot, therefore, be used to investigate the kinetics of S-phase condensation in BHK-21 that have not been synchronized by isoleucine deprivation and hydroxyurea arrest.

However, when unsynchronized proliferating cultures of BHK-21 are given caffeine plus hydroxyurea, we have not observed the very rapid formation of S-phase condensed chromosomes that is seen within 1 h when caffeine is given to BHK-21 arrested in early S-phase. Instead, unsynchronized BHK-21 cells do not start to form S-phase condensed chromosomes until after a delay of between 3 and 4 h (Fig. 3 *a*). Such a delay would be expected if S-phase condensation in an unsynchronized population first occurs, under the influence of caffeine, in cells that are blocked in late S-phase by hydroxyurea and reach the time of normal mitosis after a lag corresponding to the normal G_2 delay. Furthermore, as Fig. 3 *b* shows, when BHK-21 cells are released from prolonged hydroxyurea arrest and given caffeine and fresh hydroxyurea after 2-h recovery, the subsequent kinetics of S-phase condensation more closely resemble those seen in unsynchronized cells than those of cells not allowed time for recovery. A few S-phase condensations occur shortly after the addition of caffeine and fresh hydroxyurea, but the majority are delayed until the time of normal mitosis. Interestingly, this experiment also reveals that the addition of caffeine alone, 2 h after release from a prolonged hydroxyurea block, is capable of inducing a low frequency of S-phase condensation.

These results imply that the "premature" timing of condensation in CHO or BHK-21 cells given caffeine, as previously reported, is a consequence of the interaction of prolonged ar-

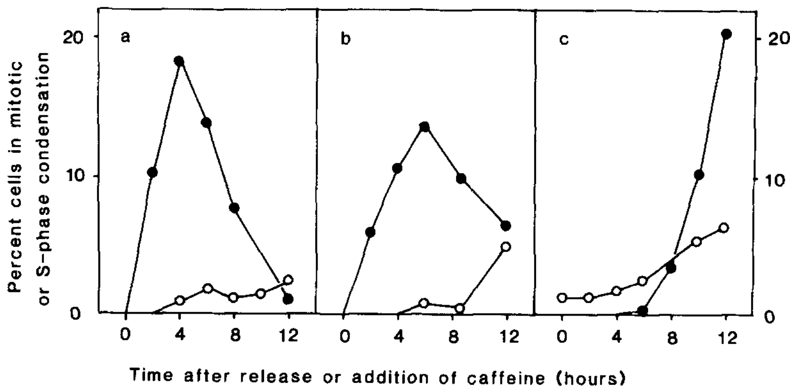

Figure 1. Kinetics of formation of S-phase condensed chromosomes in cells treated with caffeine after isoleucine depletion and hydroxyurea blockade, compared with kinetics of mitotic accumulation in cells released from blockade. After synchrony, cells were either given caffeine with hydroxyurea to determine the kinetics of S-phase condensation, or released into fresh medium to determine the kinetics of mitosis with colcemid added to accumulate mitotic cells during a 3-h period before each cell harvest. Frequencies of S-phase condensed figures (*solid symbols*) or mitotic figures (*open symbols*) were scored for (*a*) BHK-21 cells, (*b*) CHO cells, and (*c*) SVM cells.

rest of hamster cells and caffeine treatment, rather than an effect of caffeine. It appears that the critical factor is prolonged hydroxyurea arrest. We have not been able to obtain G_1 condensed chromosomes by caffeine treatment of BHK-21 or SVM cells accumulated in G_1 by isoleucine arrest alone, or by arrest with sodium butyrate. Even the induction of unscheduled DNA synthesis by UV irradiation does not produce G_1 condensed chromosomes in such cells. It also appears that this rapid interaction of caffeine and prolonged hydroxyurea arrest is specific to certain cell lines. Not only are SVM Indian muntjac cells refractory but so are human lines such as HeLa or SV40-MRC5 that (like SVM) can slowly form S-phase condensed chromosomes after replication arrest and caffeine treatment (data not shown).

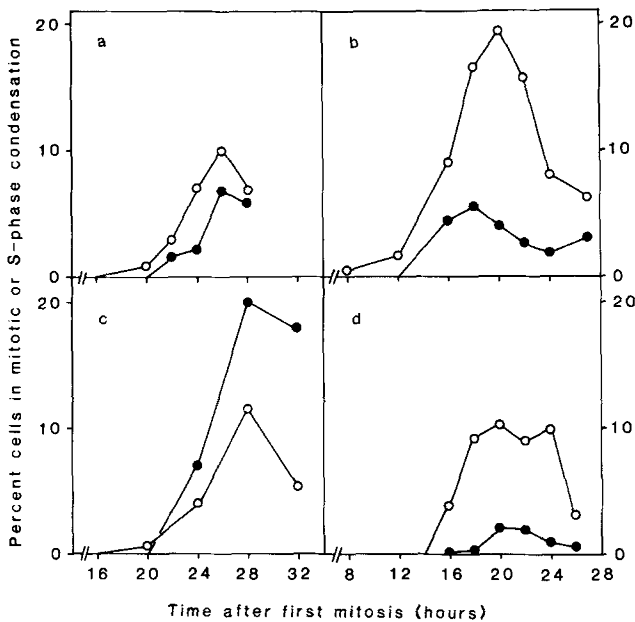

Figure 2. Kinetics of formation of S-phase condensed chromosomes in cells synchronized by mitotic arrest and treated with caffeine after UV irradiation or hydroxyurea inhibition, compared with kinetics of mitotic accumulation. Frequencies of S-phase condensed figures (*solid symbols*) were scored for (*a*) SVM cells given caffeine and hydroxyurea in G_1 8 h after synchrony; (*b*) CHO cells given caffeine and hydroxyurea in G_1 4 h after synchrony; (*c*) SVM cells given caffeine after irradiation with 2 Jm^{-2} UV in G_1 8 h after synchrony; and (*d*) CHO cells given caffeine after irradiation with 5 Jm^{-2} UV in G_1 4 h after synchrony. Frequencies of mitotic figures (*open symbols*) were scored in control populations allowed to progress towards mitosis, with colcemid added to accumulate mitotic cells during a 3-h period before each cell harvest.

Nature of Hydroxyurea-arrested BHK Cell Populations

It has been reported that extensive hydroxyurea blockade seriously deranges S-phase controls in hamster cells, allowing partial progression through the cycle and inducing endoreduplication after release from the block (22). This finding is, however, controversial; other workers deny the existence of such a phenomenon (4, 7). In BHK cells after prolonged arrest, we can find no evidence for gross disturbance of cycle control: hydroxyurea after isoleucine deprivation efficiently arrests replication, leaving an essentially G_1 population with a small number of cells with a G_2 DNA content, probably the tetraploid population endemic in BHK-21 (Fig. 4 *a*). On

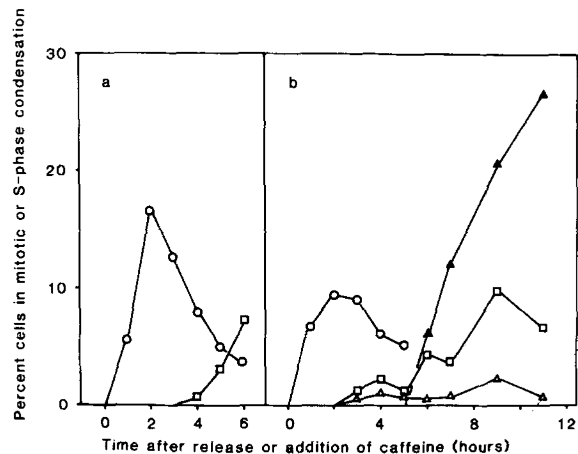

Figure 3. Kinetics of formation of S-phase condensed chromosomes in BHK-21 cells treated with caffeine after isoleucine depletion and prolonged hydroxyurea blockade, and after less continuous hydroxyurea treatment: (*a*) frequencies of S-phase condensed figures in cells given caffeine after isoleucine depletion/hydroxyurea synchrony (*open circles*), and in cells given 2 mM hydroxyurea when in random proliferating culture (*open squares*); (*b*) frequencies of S-phase condensed figures (*open symbols*) and mitotic figures (*solid symbols*) in cells given caffeine after isoleucine depletion/hydroxyurea synchrony (*open circles*), and in cells released from hydroxyurea arrest for 2 h and then given caffeine with hydroxyurea (*open squares*) or 2 mM caffeine with 0.05 μg/ml colcemid (*open triangle*).

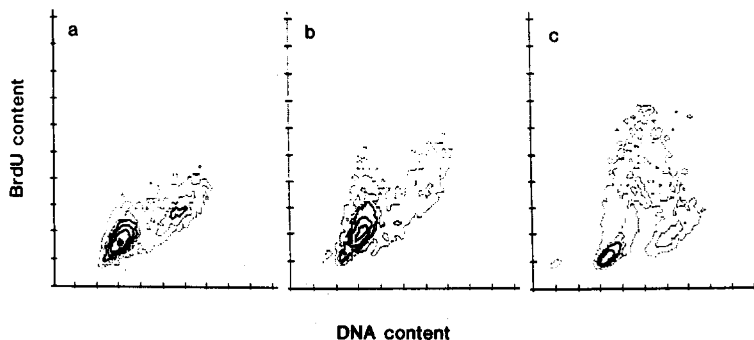

Figure 4. Flow cytometric analysis of BHK populations. Plots show frequency contours for intensity of FITC (staining for bromodeoxyuridine) and propidium (staining for DNA) fluorescence for (*a*) cells given isoleucine-free medium for 30 h, followed by medium with 2 mM hydroxyurea for 18 h, and 2 mM hydroxyurea plus BrdU for 1 h; (*b*) cells given isoleucine-free medium for 30 h, followed by medium with 2 mM hydroxyurea for 18 h, and then growth medium plus BrdU for 1 h and (*c*) unsynchronized cells given BrdU for 1 h.

release from such arrest, BHK-21 start replication from G_1 amounts of DNA (Fig. 4 *b*). The synchrony appears, by conventional criteria, to be entirely adequate.

Discussion

We conclude that caffeine's action on cycle controls can be seen clearly in hamster cells not subjected to continued and prolonged hydroxyurea arrest, and in SVM cells in all conditions. Caffeine does not, as previously supposed, accelerate progress through the cell cycle. Rather, it restores the normal cycle timing, by overcoming a restriction point in cycle progression that is otherwise imposed by the presence of unreplicated DNA. This control is clearly vital to the genetic integrity of the somatic cell. If, as has recently been suggested by Murray (13), the restraint is imposed through the phosphorylation of the *cdc2* gene product at tyrosine-15, then caffeine would act by directly or indirectly stimulating phosphatase action at this site.

Alternative explanations in terms of accumulation of cyclins, or other elements that interact with the *cdc2* product, are possible, though not yet tested. But in any case, our results imply that in hamster cells (but not SVM) prolonged replication arrest leads to the accumulation or activation of mitotic condensation factors, to the point where cells are nearly ready to enter mitosis, but are still restrained by the presence of incompletely replicated nuclei. The DNA of these nuclei remains in a very early S-phase state (Fig. 4); the cycle controls are in a state appropriate to G_2, but restrained from further advance by the presence of unreplicated DNA. Caffeine removes this restraint. It is not clear, from previous reports of caffeine's action in overriding mitotic delay after damage, or inducing S-phase condensation, whether its effect is dependent on protein synthesis as proposed by Schlegel and Pardee (23) or occurs at a posttranslational level as proposed by Rowley (20). Our results would be consistent with an accumulation, in hydroxyurea-treated cells, of mitosis-specific mRNAs as suggested by Schlegel et al. (25), or with other possible mechanisms of altering cycle controls.

This priming of the mitosis condensation factors appears, however, to be a consequence of prolonged hydroxyurea blockade, not of cycle arrest as such. In addition to the evidence presented above, rapid condensation has been reported in CHO cells given caffeine after a long hydroxyurea blockade without previous isoleucine deprivation (1). However, the disturbance of cell cycle controls caused in hamster cells by prolonged arrest is far from total, and is (as shown in Fig. 3 *b*) largely reversible within a few hours of release from hydroxyurea. In this context, it is notable that rapid "premature" condensation of S-phase nuclei in *tsBN2* mutants was observed in cells raised to the restrictive temperature immediately after prolonged hydroxyurea arrest (15), but did not occur in cells left to recover after hydroxyurea synchrony (21).

It is intriguing that caffeine-treated cells are in some ways analogous to early embryonic cells, before the midblastula transition; in such cells the inhibition of replication by aphidicolin does not prevent subsequent cycles of cell division in *Xenopus* embryos (9) or mitotic condensation in *Drosophila* embryos (19). It may be relevant that early embryo cells have many small replicons (3, 10), and a well-established action of caffeine on mammalian cells is an increase in the number of functioning replicons (17). The analogy is not, however, complete: embryo mitotic cycles, when uncoupled from division, are longer than normal (19). It is not known whether caffeine affects early embryo cells in which replication is inhibited.

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