Prolonged arrest of mammalian cells at the G1/S boundary results in permanent S phase stasis

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Summary

Mammalian cells in culture normally enter a state of quiescence during G1 following suppression of cell cycle progression by senescence, contact inhibition or terminal differentiation signals. We find that mammalian fibroblasts enter cell cycle stasis at the onset of S phase upon release from prolonged arrest with the inhibitors of DNA replication, hydroxyurea or aphidicolin. During arrest typical S phase markers remain present, and G0/G1 inhibitory signals such as $p21^{WAF1}$ and p27 are absent. Cell cycle stasis occurs in T-antigen transformed cells, indicating that p53 and pRB inhibitory circuits are not involved. While no DNA replication is evident in arrested cells, nuclei isolated from these cells retain measurable competence for in vitro replication. MCM proteins are required to license replication origins, and are put in place in nuclei in G1 and excluded from chromatin by the end of replication to prevent rereplication of the genome. Strikingly, MCM proteins are strongly depleted from chromatin during prolonged S phase arrest, and their loss may underlie the observed cell cycle arrest. S phase stasis may thus be a 'trap' in which cells otherwise competent for S phase have lost a key component required for replication and thus can neither go forward nor retreat to G1 status.

Key words: S phase stasis, S phase, Mammalian, MCM proteins, Hydroxyurea, Aphidicolin

Introduction

The integrity of the eukaryotic genome requires several layers of control to ensure that replication of nuclear DNA occurs once and only once during a cell cycle. Evidence from cell fusion experiments (Rao and Johnson, 1970) and from in vitro replication assays of isolated nuclei (Krude et al., 1997) has demonstrated that G1 nuclei are competent to engage in replication if supplied factors present in S phase cells, but that G2 nuclei are incompetent to replicate. These results suggest that a sequential series of controls during the cell cycle 'license' replication during G1, then activate replication during S phase, and then inactivate the licensing of the replication process during G2.

For licensing to occur, proteins important to the initiation of replication are positioned at origins of replication in the nucleus during G1 under the control of the origin recognition complex (ORC). Both Cdc6/18 and Cdt1 are recruited to origins and in turn load the MCM proteins (Blow and Tada, 2000; Coleman et al., 1996; Donovan et al., 1997; Gillespie and Blow, 2000; Maiorano et al., 2000; Nishitani et al., 2000; Tanaka et al., 1997). The MCM proteins (MCM 2-7), a complex of related proteins (Tye, 1999) must be put in place during G1 to initiate replication. They are then lost from the chromatin-bound fraction as replication proceeds (Aparicio et al., 1997; Hendrickson et al., 1996; Krude et al., 1996; Liang and Stillman, 1997), and must be replaced by Cdc6/18 and Cdt1-dependent processes in the following G1 for DNA replication to reinitiate.

The onset of S phase is triggered by the activity of the protein kinases Cdc7-Dbf4, Cdk2-cyclin E and Cdk2-cyclin A (Jiang et al., 1999a; Johnston et al., 2000; Zou and Stillman, 1998). These protein kinases have two discrete functions, activating the replication process and simultaneously eliminating Cdc6 activity by marking it for export from the nucleus in higher eukaryotes (Jiang et al., 1999a; Petersen et al., 1999; Saha et al., 1998). The kinase activity thus initiates S phase at the same time that it blocks reinitiation of replication. By the onset of S phase, Cdt1 is largely gone (Nishitani et al., 2001). Further, geminin, a recently described protein is produced during S phase (McGarry and Kirschner, 1998), and suppresses residual Cdt1 (Nishitani et al., 2001; Tada et al., 2001; Wohlschlegel et al., 2000). As a result, for replication, the cell is dependent on MCM that has been loaded at origins during G1, consistent with the licensing function of the MCMs that permits only a single round of replication. If MCMs were lost from an origin prior to replication, the associated origin should, in principle, be unable to initiate replication. Cdc6 and Cdt1 are thus limiting factors whose controlled loss from the nucleus during S phase prevents reinitiation of replication until the next cell cycle at origins that have already fired.

During S phase, replication can cease in response to DNA damage or stress to the replication process. Stress can be induced by hydroxyurea or by aphidicolin, two drugs with different mechanisms of action (Ikegami et al., 1978; Timson, 1975) that suppress migration of the replication fork without provoking DNA damage. Stress to the replication process induces arrest through mechanisms different from those invoked by DNA damage. DNA damage induces ATM protein kinase as a critical intermediate blocking S phase progression, but stress does not (Gottifredi et al., 2001). DNA-damage-induced arrest initiates a p53 and $p21^{WAF1}$ response (Dulic et

al., 1994; el-Deiry et al., 1993), with $p21^{WAF1}$ specifically binding to and inhibiting PCNA, the auxiliary factor for DNA polymerases δ and ε (Li et al., 1994; Waga et al., 1994). In contrast, response to replicative stress arrests all cells regardless of p53 status (Linke et al., 1996) and is not accompanied by $p21^{WAF1}$ induction (Gottifredi et al., 2001). Additionally, cell cycle progression following release from HU block occurs regardless of the presence of p53 (Gottifredi et al., 2001).

Normally, cells reinitiate S phase rapidly and synchronously following release from hydroxyurea (HU) or aphidicolin, and such drug treatments are routinely used to generate cell synchrony at the G1/S phase boundary. However, we have noted in our work that a portion of the cell population of a number of mammalian cells does not reinitiate replication following arrest for approximately one cell cycle, but remains arrested with 2N DNA content. Here, we have addressed the status of the arrested cells, and found that all cycling cells have the capacity to permanently arrest following release from HU or aphidicolin, and that they appear to be arrested in S phase. There is no indication that arrest is due to DNA damage. However, we find that the MCM proteins are partially displaced from the chromatin-bound fraction during prolonged arrest. This loss is not sufficient to prevent at least partial replication of in vitro isolated nuclei. Whereas intact cells cannot replicate following release from prolonged S phase block, nuclei isolated from arrested cells remain competent to initiate DNA replication in vitro after 60 hours of arrest. We conclude that initiation of replication is specifically suppressed following prolonged arrest, perhaps in response to partial displacement of MCM proteins, generating a stable static population.

In mammalian cells, quiescence typically occurs in the G1 phase of the cell cycle. When a quiescent state stems from serum starvation, contact inhibition, senescence or differentiation, it is typically accompanied by induction of p53, $p21^{WAF1}$ and/or p27 (Johnson et al., 1998; Kato et al., 1994; Polyak et al., 1994; Reynisdottir et al., 1995; Stein et al., 1999). The prolonged cell cycle arrest in S phase that we report here is thus novel, as it is independent of the cyclin-dependent kinase inhibitors $p21^{WAF1}$ and p27 and appears to involve indefinite suppression of the S phase replicatory mechanism. The simplest interpretation of these results is that cells become trapped in a state we call S phase stasis by irretrievable loss of MCM proteins from the nuclear matrix, and thus cannot go forward to replicate nor return to G1.

Materials and Methods

Cell culture and synchronization

HeLa, REF-52 (rat embryo fibroblasts), and their SV40 large T antigen transformed derivatives (TAG) (Perry et al., 1992) were cultured as monolayers in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek Israel). To maintain IMR 90 cells, the medium was supplemented with 20% fetal calf serum (Hyclone Laboratories, Logan, UT). All cells were maintained in a humid incubator at 37°C in a 5% CO_2 environment.

G0 synchronization was obtained by growing REF-52 cells to confluency and maintaining them in contact inhibition (determined by absence of mitotic cells) for at least 24 hours. Cells were then released from contact inhibition by replating in fresh medium at a dilution of 1:5.

To synchronize REF-52 cells at the G1/S boundary, contact inhibited cells were replated in fresh medium and 8 hours later were exposed to aphidicolin for 15 hours. Aphidicolin, hydroxyurea, nocodazole and caffeine were applied at 10 μM, 2 mM, 0.25 μg/ml and 2 mM respectively. Aphidicolin and nocodazole were prepared as stock solutions in DMSO at 10 mM and 1 mg/ml, respectively. Hydroxyurea and caffeine were prepared as 200 mM stock solutions in DMEM containing 10% fetal bovine serum.

Flow cytometric analysis

For flow cytometry, attached cells were collected by trypsinization, pooled with non-attached cells, centrifuged and resuspended in PBS, then fixed by the addition of methanol to 90% at –20°C. After 10 minutes fixation, cells were pelleted, then resuspended and stored in PBS with 0.04% sodium azide. For flow cytometry fixed cells were washed with PBS and resuspended in 4 mM sodium citrate containing 30 U/ml RNase A, 0.1% Triton X-100, and 50 μg/ml propidium iodide and incubated for 10 minutes at 37°C. Sodium chloride was then added to 138 mM. Data were collected using a FACScan apparatus (Becton Dickinson, San Jose, CA) and results were analyzed with Becton Dickinson Cell Quest software. For each sample, 10,000 events were collected and aggregated cells were gated out.

Protein extraction and Cdk2/Cdc2 protein kinase assay

REF-52 cells were collected by trypsinization, washed with cold PBS, and cell lysates were prepared in lysis buffer: 50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 5 mM EGTA, 0.1% NP-40 containing 4 mM Pefabloc, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 60 mM β-glycerophosphate, 50 mM NaF, and 0.5 mM sodium vanadate, as previously described (Andreassen and Margolis, 1994).

For kinase assays, 50 μg of each extract was incubated with 25 μl of protein A-Sepharose 4B beads for 30 minutes at 4°C to preclear proteins that bind nonspecifically to the beads. 4.0 μl of rabbit anti-Cdk2 antiserum (kind gift of R. Fotedar, Institut de Biologie Structurale, Grenoble, France) or rabbit anti-Cdc2 antiserum (Andreassen and Margolis, 1994) was added to the extract for 1 hour at 4°C, followed by addition of 50 μl of protein A-Sepharose beads, and incubation for 1 hour at 4°C. The resulting immune complex was washed three times with lysis buffer. The pellet was washed once, then resuspended, in 50 μl of kinase buffer (50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mg/ml BSA) containing 1 μg calf thymus histone H1 (Roche Diagnostics), 30 μM ATP, and 5 μCi of [γ-^{32}P]ATP (Amersham). The H1 kinase assay was carried out for 30 minutes at 37°C and was terminated by the addition of polyacrylamide sample buffer. Samples were then resolved by SDS-PAGE on 12% polyacrylamide gels (19:1 ratio of acrylamide to *bis*-acrylamide) (Andreassen and Margolis, 1994). Autoradiographs were prepared by exposure to Hyperfilm-MP (Amersham).

Chromatin and nuclear matrix fractionation

The chromatin/nuclear matrix fractionation assay was performed essentially as described (Jiang et al., 1999b) with minor modifications. Cells were trypsinized and washed with 1 ml ice-cold PBS and then lysed in 500 μl of 10 mM Hepes pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 100 mM NaCl, 300 mM sucrose, containing 4 mM Pefabloc, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 6 mM β-glycerophosphate, 5 mM NaF and 0.5 mM sodium vanadate for 25 minutes. After low speed centrifugation (1500 ***g***, 5 minutes, 4°C) the nuclei were extracted once more with 250 μl of extraction buffer for 10 minutes, aliquoted and snap frozen in liquid nitrogen. The supernatants of the first spins were respun at 16,000 ***g*** for

5 minutes and the soluble fractions were aliquoted and snap frozen.

Immunoblotting

To prepare immunoblots, 20-30 μg of total protein were resolved on polyacrylamide gels and proteins were transferred to nitrocellulose sheets using semi-dry blotting apparatus. After blocking with 5% nonfat milk in TNT buffer (25 mM Tris, pH 7.5, 150 mM sodium chloride, and 0.05% Tween 20) nitrocellulose membranes were incubated overnight with primary antibodies in TNT containing 5% nonfat milk. Nitrocellulose membranes were then washed, and incubated for 1 hour with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies diluted in TNT with 5% nonfat milk. Development of the protein-antibody complex was performed using enhanced chemiluminescence according to manufacturer's instructions (Pierce, Rockford, IL).

Preparation of nuclei

Nuclei were prepared essentially as described (Krude et al., 1997). Cells from four 15 cm plates were trypsinised, pooled and washed once with ice cold PBS and once with ice-cold hypotonic buffer. Pelleted nuclei (400 ***g***, 4 minutes, 4°C, Eppendorf centrifuge) were resuspended in 1 ml of hypotonic buffer (20 mM K-Hepes pH 7.8, 5 mM potassium acetate, 0.5 mM $MgCl_2$, 0.5 mM DTT), left on ice for 10 minutes and then disrupted with 30 strokes in a Dounce homogenizer using a loose fitting pestle. After centrifugation (1500 ***g***, 5 minutes, 4°C), pelleted nuclei were washed three times with PBS and resuspended in 0.1 ml of PBS containing 5% DMSO, aliquoted and snap frozen in liquid nitrogen.

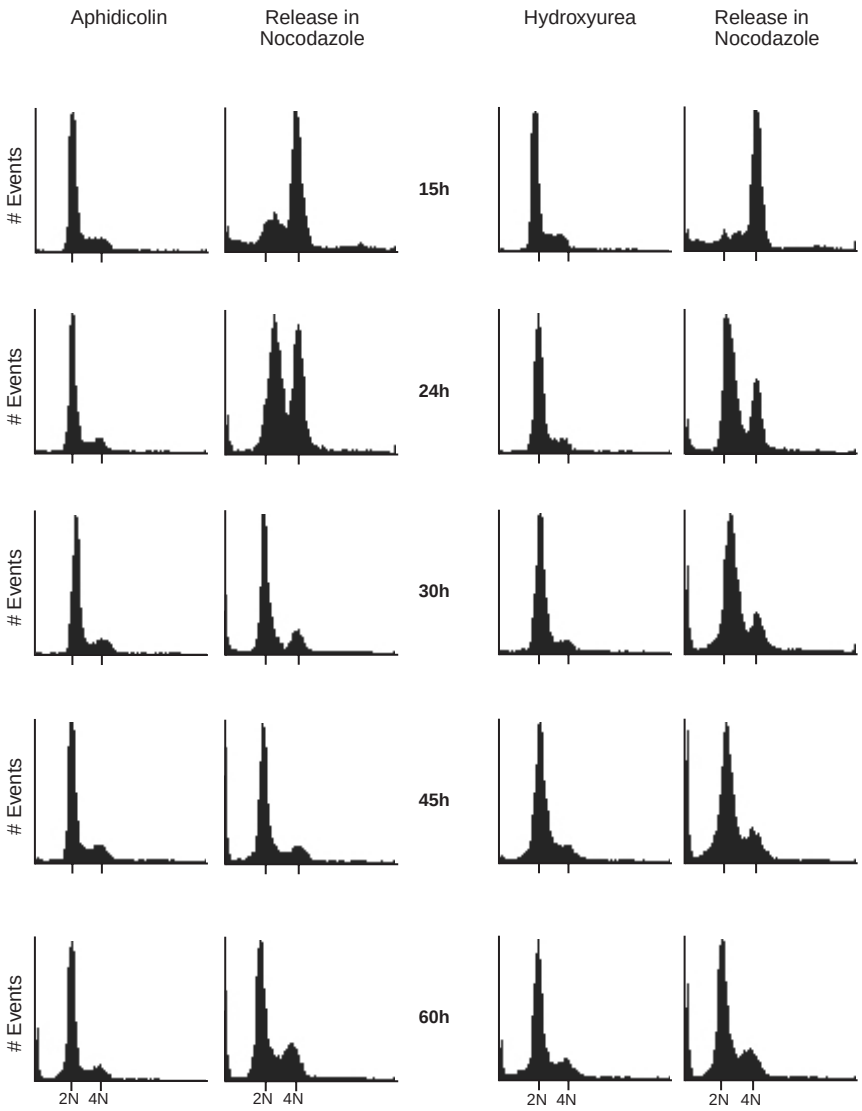

Fig. 1. Random cycling REF-52 cells do not recover from hydroxyurea or aphidicolin block after 30 hours of exposure. Random cycling REF-52 cells were treated with aphidicolin (10 μM) or hydroxyurea (2 mM) for the indicated times, then released for 24 hours into nocodazole (0.25 μg/ml), and finally subjected to FACscan analysis. Failure of cells exposed to drug for 30 hours or more to replicate upon release is reflected by the absence of cells arrested in 4N by nocodazole.

In vitro DNA synthesis reaction

In vitro DNA synthesis was performed on isolated nuclei as described (Krude et al., 1997). 20 μl of nuclei were incubated in a final volume of 50 μl for each sample in 40 mM K-Hepes pH 7.8, 7 mM $MgCl_2$, 3 mM ATP, 0.1 mM each of GTP, CTP, UTP, dATP, dGTP and dCTP, 0.25 μM biotin-16dUTP, 0.5 mM DTT, 40 mM creatine phosphate and 5 μg of phosphocreatine kinase for 2 hours at 37°C. Reactions were stopped by diluting with 450 μl of PBS and nuclei were fixed by adding 500 μl of 8% paraformaldehyde. After 5 minutes at room temperature, nuclei were pelleted (8 minutes, 2000 ***g***, Eppendorf centrifuge). The nuclei were then resuspended in 100 μl of 30% sucrose in PBS and spun onto poly-lysine-coated coverslips.

Immunofluorescence microscopy

For in vivo determination of DNA replication, cells were pulsed for 30 minutes with 10 μM bromodeoxyuridine (BrdU) prior to being harvested. For BrdU labelling, cells were grown on poly-lysine-coated glass coverslips and then fixed for 20 minutes at 37°C with 2% paraformaldehyde in PBS, washed with PBS, permeabilized with 0.2% Triton X-100 in PBS for 3 minutes, and washed again with PBS. Coverslips were then incubated for 30 minutes in 2N HCl/0.5% Triton X-100, washed with PBS and neutralysed with 0.1 M sodium tetraborate, pH 8.5, for 5 minutes. After washing with PBS, cells were incubated with FITC-conjugated anti-BrdU (Becton Dickinson, San Jose, CA) diluted 30-fold in PBS/0.5% Tween 20/1% bovine serum albumin. In all cases, cells were counterstained with propidium iodide (25 μg/ml) containing RNase A (1 μg/ml).

For observation of in vitro replication, isolated nuclei were labeled with FITC-conjugated streptavidin (Vector Laboratories, Burlingame, CA), diluted 50-fold in PBS/0.5% Tween 20/1% bovine serum albumin, to detect incorporation of biotin-16-dUTP. Nuclei were counterstained with propidium iodide (25 μg/ml) containing Rnase A (1 μg/ml).

For microscopy, coverslips were mounted as previously described (Andreassen et al., 1991) and observed using an MRC-600 Laser Scanning confocal apparatus (Bio-Rad Microscience Division, Hemel Hempstead, UK).

Results

Both transformed and non-transformed cells permanently arrest following release from prolonged exposure to S phase block

In previous work using S phase arrest procedures, we have observed that a portion of a randomly cycling cell population usually fails to recover from arrest. To determine whether the entire population had the capacity to enter into stasis, we analyzed this effect systematically by exposing REF-52 cells to hydroxyurea (HU) or aphidicolin for varying periods of time, then assaying the capacity of the cells to recover from the block.

REF-52 cells were blocked at the G1/S transition by exposure to either 10 μM aphidicolin or 2 mM HU for different times, as indicated (Fig. 1), then released into nocodazole for 24 hours. Cells that were capable of progressing from the S phase block to mitosis would then collect as a 4N population. The result that we obtained was striking. After as little as 24 hours exposure to either drug, at least 50% of the randomly cycling population was unable to recover and progress in the cell cycle (Fig. 1A). HU appeared to cause a more rapid progression to arrest, as the majority of HU exposed cells could not recover after 24 hours exposure. However, despite the different mechanisms of action of the two drugs, the outcome with respect to entry into arrest was the same.

Many cell cycle checkpoint controls involve either p53 or pRB, but the progression of cells to stasis was independent of p53 or pRB controls, and thus not equivalent to entry into G1 quiescence. SV40 large T-antigen suppresses the checkpoint functions of both p53 and pRB (Ludlow et al., 1989; Zhu et al., 1991). TAG cells are a REF-52-derived cell line transformed by large T-antigen. When we performed a parallel analysis on TAG cells, exposing cells to either HU or aphidicolin in the presence of nocodazole, we found essentially the same capacity to permanently arrest as the REF-52 parental line after 30 hours exposure to either HU or aphidicolin (Fig. 2). We note that at the earliest time of exposure (15 hours), a substantial number of TAG cells progress past 4N or are less than 2N and clearly dying. This effect results from the failure of TAG cells to arrest in tetraploid G1 following nocodazole exposure (Andreassen et al., 2001). These cells rapidly progress towards aneuploidy and apoptosis. The observed aneuploidy and death are independent of S phase arrest, as longer times of exposure actually protect cells against these outcomes by maintaining S phase blocked and released cells in 2N arrest.

REF-52 cells were also released from G0 block and exposed to either HU or aphidicolin for varying times before release into nocodazole. The cells arrested, but this effect occurred after a longer time of exposure to drug than was observed in randomly cycling cells, presumably because of the longer time required for cells to reach the G1/S boundary from G0 than from the random cycle. Arrest was typically nearly complete after 45 hours of exposure to either drug (Fig. 3). Further, the observed arrest persisted over a period of 7 days following release from aphidicolin S phase block (Fig. 4A), and failure of cell cycle progression correlated with results of proliferation curves following release from aphidicolin over a period of 2 days (Fig. 4B).

The entry into arrest was not unique to rat REF-52 and TAG cells. Parallel results were obtained with primary human fibroblast (IMR-90) cells that were either randomly cycling (Fig. 5) or synchronized in G0 (data not shown) prior to drug treatment, and arrest in these cells also followed exposure to either HU or aphidicolin.

Permanently arrested cells maintain continuing S phase status

We wanted to understand the nature of the blocked state that these cells exhibited after prolonged exposure to S phase arrest. Typically, when mammalian cells enter into a quiescent state, it is in G1 phase. Exit from the cell cycle may be either temporary, such as during serum starvation or contact inhibition, or permanent, as typified by senescence. We therefore asked whether these cells reverted to a G1 or G0 state in prolonged arrest, or if they remained in S phase but lost the capacity to replicate.

In order to determine the cell cycle status of the arrested cells, we exposed REF-52 cells, released from contact inhibition, to aphidicolin for 15, 30, 45 or 60 hours, then

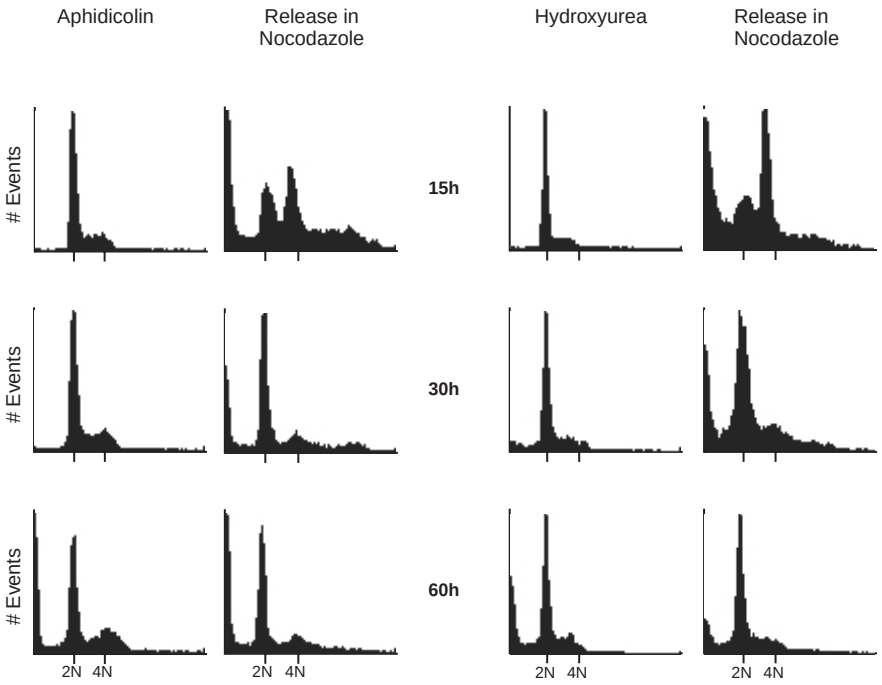

Fig. 2. Failure to replicate following prolonged exposure to hydroxyurea or aphidicolin is not dependent on p53 or pRB function. T-antigen-transformed REF-52 cells (TAG) were subjected to analysis as for REF-52 (Fig. 1). FACScan analysis of random cycling TAG cells treated with aphidicolin (10 μM) or hydroxyurea (2 mM) for the indicated times, then released for 24 hours into nocodazole (0.25 μg/ml), shows failure to progress in the cell cycle after 30 hours of treatment.

prepared cell extracts to assay for the abundance of different cell cycle markers. All the markers that we assayed were consistent with a continuing S phase status in the prolonged arrest (Fig. 6A). PCNA, a DNA polymerase cofactor required for S phase progression, was equally abundant at 30, 45 and 60 hours. Other proteins required for S phase progression, such as cyclin A and Cdk2, also were not diminished. In contrast, proteins required for G1 progression (Cdk4) or S phase entry (cyclin E) had diminished at later time points of arrest. The cyclin-dependent kinase inhibitors of G1 progression, p27 and p21, were diminished or nearly absent in the arrested state. The relative absence of p21, which is transactivated by p53 and can provoke S phase arrest in response to DNA damage (Li et al., 1994; Waga et al., 1994), is consistent with the parallel induction of arrest independent of the p53 status of the treated cells. We conclude from this evidence that the cells remain in S phase and do not revert to G1 or G0 status when they arrest.

Cdk2, the cyclin-dependent kinase required for S phase progression (Tsai et al., 1993), showed activity levels consistent with continuing S phase status of the arrested cells (Fig. 6B). Interestingly, Cdc2, the cyclin-dependent kinase required for G2/M progression (Draetta and Beach, 1988), was more abundant at the 45 hours arrest point than at 30 hours (Fig. 6A), and also showed higher activity levels (Fig. 6B) at this time point. Despite the increase in Cdc2 activity, there was no physical evidence for mitotic entry of the blocked cells. In fact, despite the presence of Cdc2 activity, attempts to induce checkpoint override from S phase with caffeine (Schlegel and Pardee, 1986) did not induce mitotic entry in either 45 or 60 hours arrested cells (data not shown).

Progression in S phase is accompanied by the continuous loss of the MCM family of proteins from the chromatin-bound fraction (Aparicio et al., 1997; Hendrickson et al., 1996; Krude et al., 1996; Liang and Stillman, 1997). The MCM proteins are loaded onto chromatin by the G1-specific factors Cdc6/18 and Cdt1 (Blow and Tada, 2000; Coleman et al., 1996; Donovan et al., 1997; Gillespie and Blow, 2000; Maiorano et al., 2000; Nishitani et al., 2000; Tanaka et al., 1997), are absolutely required for replication, and are lost from chromatin in the late cell cycle to prevent re-replication of the genome (Tye, 1999). We have followed the status of two of the members of the MCM protein complex, MCM3 and MCM4, during S phase blockage, and have consistently found a partial loss from the chromatin-bound fraction during prolonged drug exposure (Fig. 6A). MCM presence on chromatin in S phase is partially controlled by an interplay with geminin (Wohlschlegel et al., 2000). After Cdt1 helps load the MCM proteins on chromatin in G1, the increasing abundance of geminin during S phase suppresses Cdt1 and permits MCM loss (Nishitani et al., 2001; Wohlschlegel et al., 2000). The observed loss of MCM in replication block might be due to the persistence of geminin if it continued to be translated during the S phase block. We therefore tested for the relative levels of geminin in cells blocked in S phase for varying amounts of time. Consistently, we found that geminin levels persisted and even increased as the cells became permanently arrested (Fig. 6C). We found that geminin was largely in the chromatin unbound fraction (Fig. 6C), indicating that there was little change in geminin-binding status on chromatin as cells entered arrest.

Do arrested cells retain S phase competence?

Our evidence indicates that cells that remain arrested following release from prolonged exposure to S phase inhibitors remain in an S phase state. From FACscan analysis, we do not observe the majority of the population progressing from an apparent 2N status. There is, however, the possibility that a small degree of replication can occur in these cells. To assay for a low level of replication, we exposed cells to BrdU over a 5 hour period of time following release from aphidicolin arrest. After release from contact inhibition, REF-52 cells were exposed to

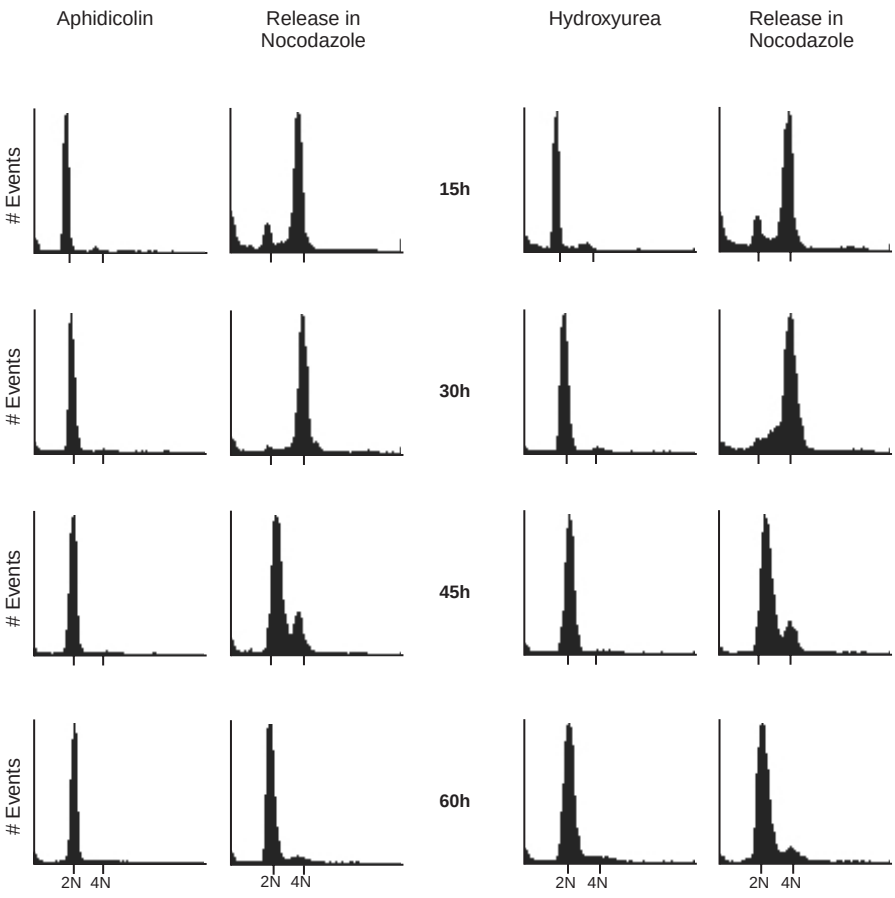

Fig. 3. REF-52 cells released from G0 state require longer drug exposure than random cycling cells to permanently arrest. REF-52 cells were synchronized in G0, as described in Materials and Methods, treated with aphidicolin (10 μM) or hydroxyurea (2 mM) for the indicated times, and then released for 24 hours into nocodazole (0.25 μg/ml). FACScan analysis shows failure to replicate after at least 45 hours of S phase arrest.

aphidicolin for various times, then assayed by immunofluorescence for BrdU uptake following aphidicolin release (Fig. 7A). By 15-20 hours after release from contact inhibition, cells are not yet in S phase, and lack of BrdU uptake in the population treated for 15 hours serves as a negative control. By contrast, the 30 hours population is fully competent to recover and proceed to mitosis, serving as a positive control for the sensitivity of the assay. In the immunofluorescence assay, two-thirds of the population competent to replicate was positive. In comparison with these values, cells released after 45 hours in aphidicolin had a low but statistically significant subpopulation capable of at least some replication. After 60 hours of aphidicolin, there was no perceptible incorporation of BrdU. We conclude that the majority of cells after 45 hours, and all of the cells after 60 hours of aphidicolin treatment show no evidence of DNA replication.

We next asked whether the failure to replicate reflected the loss of competence to initiate replication or whether a factor was present in nuclei that actively interfered with initiation of replication, perhaps in response to decreased levels of bound MCM protein. To address this issue, we isolated nuclei from cells treated for varying periods of time with aphidicolin following release from contact inhibition, then assayed their capacity to replicate in an in vitro assay system (Fig. 7B,C) (Krude et al., 1997) using a biotin-dUTP incorporation immunofluorescence assay (Krude et al., 1997). The result was striking. Whereas nuclei treated for 15 hours, not yet in S phase, did not replicate (Fig. 7B,C), nuclei from cells treated with aphidicolin for 30 or 60 hours then released showed sufficient replication to be positive by immunofluorescence assay. We conclude that prolonged exposure to S phase arrest provokes an active and durable suppression of S phase recovery, rather than total loss of replication competence.

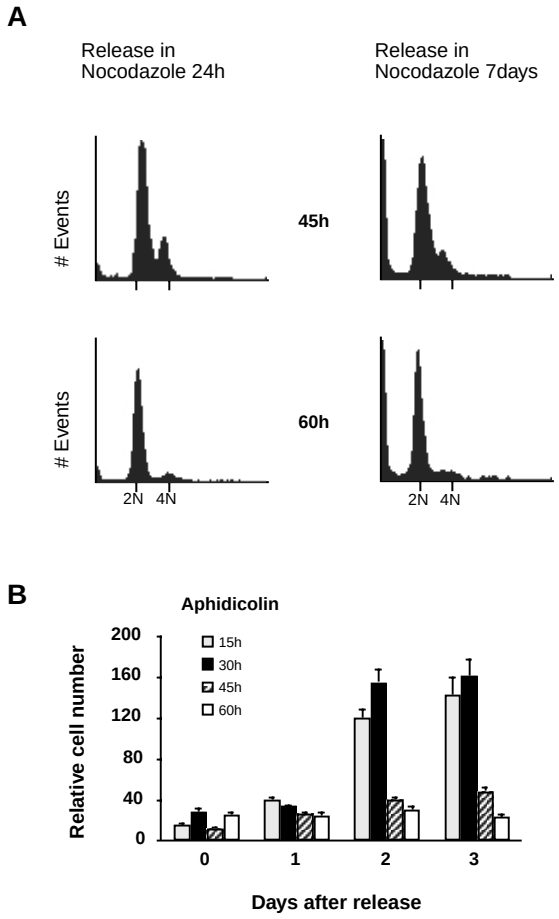

Fig. 4. REF-52 cells remain arrested for at least 7 days following release from prolonged treatment with aphidicolin or hydroxyurea. (A) FACScan analysis of G0 synchronized REF-52 cells treated with aphidicolin (10 μM) for the indicated times and then released for 24 hours or for 7 days in nocodazole (0.25 μg/ml). Cells do not replicate and progress to mitosis during this period of time, as shown by the absence of 4N population. (B) Cell counts confirm the absence of cell proliferation after aphidicolin treatment for times equal to or greater than 45 hours. G0 synchronized REF-52 cells treated with aphidicolin for the indicated times were either harvested (time 0) and counted or were released into drug-free medium. Cell counts were then taken every 24 hours over 4 days in the released population. For counting, cells that had initially been plated at equal density were trypsinized, resuspended in 1 ml PBS and the number of cells present in 20 μl was determined using a Thoma cell counting chamber. Each value is an average of at least 8 counts, and error bars represent the corresponding standard deviations.

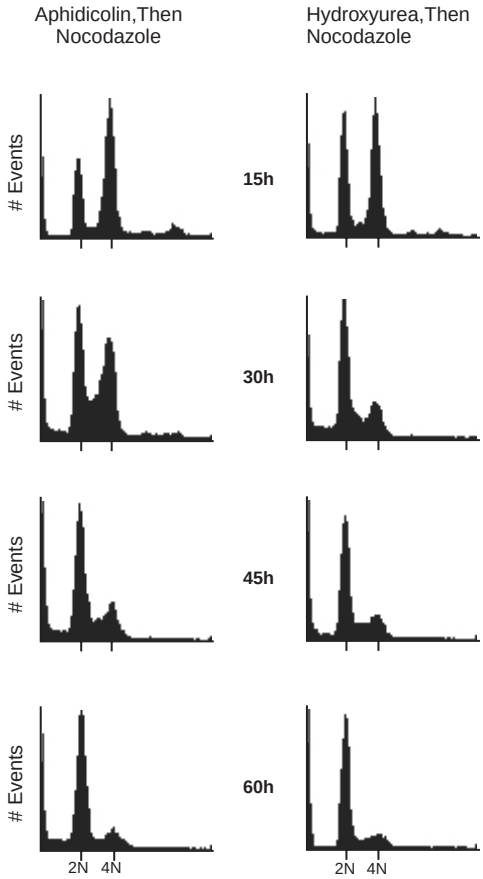

Fig. 5. Human non-transformed IMR-90 fibroblasts permanently arrest following prolonged S phase arrest, as indicated by FACScan analysis of random cycling cells treated with aphidicolin (10 μM) or hydroxyurea (2 mM) for the indicated times and then released for 24 hours into nocodazole (0.25 μg/ml). In parallel with results from REF-52, IMR-90 cells released from G0 arrest require longer than random cycling cells to permanently arrest (data not shown).

Discussion

When cells arrest in the cell cycle and enter a quiescent state, they typically exit from G1 into G0 (Pardee, 1989). The G0 state is characterized by the absence of S phase markers and the presence of G1 cyclin-dependent kinase inhibitors such as p21^{WAF1} and p27. When these inhibitors are expressed, they suppress Cdk activity required for progression to S phase. We have found that mammalian cells arrested for a prolonged period of time at the G1/S boundary by drugs that interfere with initiation of DNA replication become permanently arrested. Surprisingly, although they are unable to proceed in the cell cycle, they continue to exhibit S phase markers, and show no evidence of re-establishing G1 markers or cyclin-dependent kinase inhibitors. Our results therefore lead us to conclude that cells maintain prolonged S phase arrest while remaining metabolically in S phase.

The arrested state, which we call S phase stasis, arises following prolonged S phase inhibition with either HU or aphidicolin. The two drugs have different mechanisms of action. HU blocks ribonucleoside diphosphate reductase thus blocking production of the deoxyribonucleotide required for replication (Timson, 1975), while aphidicolin specifically inhibits nuclear DNA synthesis by suppressing DNA polymerase α (Ikegami et al., 1978). Neither drug creates DNA damage, and both are readily reversible following short-term exposure. The absence of DNA damage is in accord with the absence of requirement for p53 or pRB to mediate S phase arrest during drug exposure. Arrest in S phase requires active suppression through transactivation of suppressors, direct binding of suppressors to replication machinery, and phosphorylation of critical elements of replication control (Kelly and Brown, 2000). All of these inhibitory activities are reversible following resolution of the stress that caused the replication arrest. S phase stasis is unlikely to result from a permanent alteration of one of these transient events.

Since we observe depletion of chromatin-bound MCM in arrested cells, it is conceivable that S phase stasis arises in response to changes in MCM or in the activity of the proteins that maintain them in chromatin-bound status. Chromatin-associated MCM proteins are essential for replication, and are displaced from chromatin as S phase progresses. As a

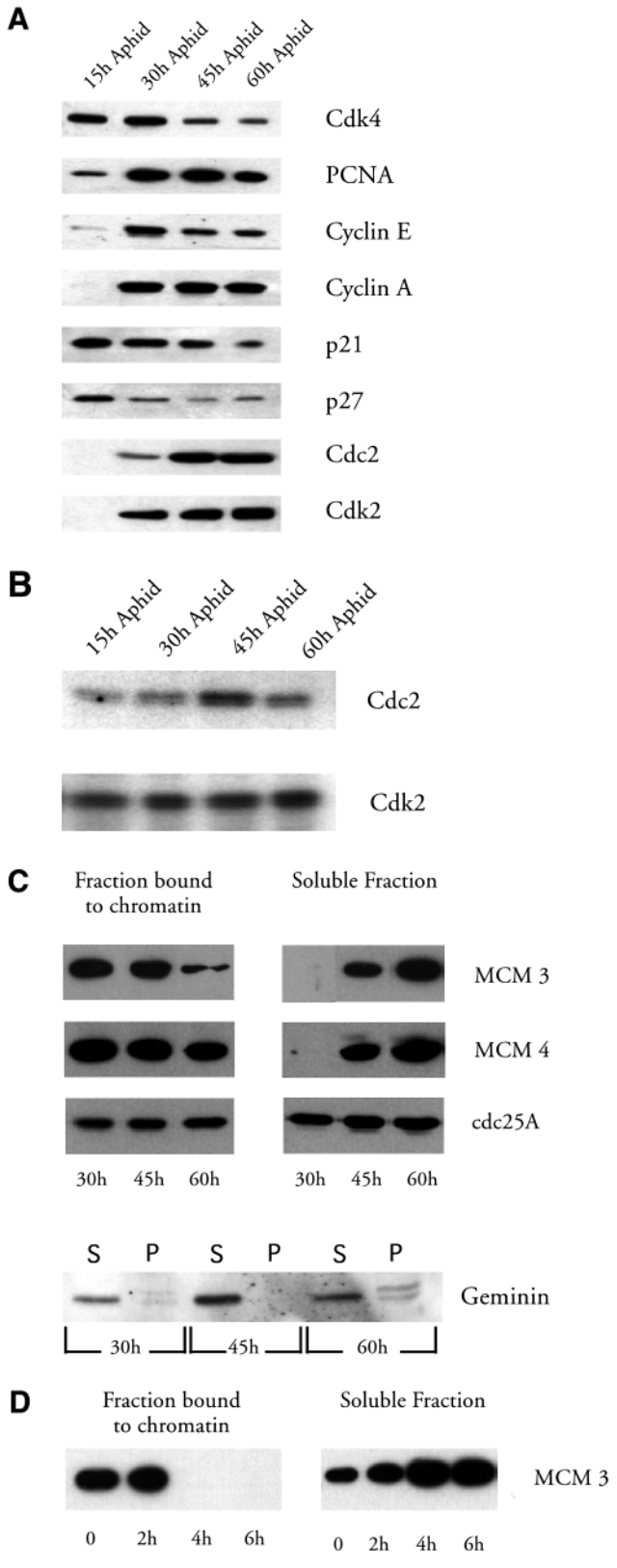

Fig. 6. REF-52 cells induced to permanently arrest by aphidicolin contain markers and activity consistent with S phase arrest. (A) Protein expression levels. REF-52 cells released from G0 arrest were treated with aphidicolin (10 μM) for the indicated times, then harvested. To determine expression levels of various cell cycle proteins, samples were subjected to SDS-PAGE, and then exposed to the appropriate antibodies for western blots. (B) Cyclin-dependent kinase activity. The kinase activities of Cdk2 and Cdc2 were determined in similar cell extracts, following specific immuneprecipitation of the enzymes, using histone H1 as substrate. Autoradiographs of ^{32}P incorporation from [γ-^{32}P]ATP are shown. (C) Release of MCM3 and MCM4 from the nuclear matrix fraction during prolonged arrest in S phase. MCM proteins were analyzed for their localization to the nuclear matrix when treated with aphidicolin. For analysis of prolonged S phase block, G0 synchronized REF-52 cells were treated with aphidicolin for the times indicated. Immunoblots of MCM3 and MCM4 proteins in chromatin-bound and soluble fractions are shown. The soluble fractions contain both cytoplasmic and soluble nuclear proteins. Cdc25A, which remains constant in the two fractions, was used as a loading control. (D) The progressive release of MCM3 from the nuclear matrix fraction to the soluble fraction during normal S phase progression is shown as a control. For this analysis, REF-52 cells were synchronized at the G1/S boundary by 30 hours treatment with aphidicolin, released into drug-free medium, and then extracts were prepared every 2 hours during S phase recovery.

consequence, G2 nuclei are unable to replicate in an in vitro replication system unless MCM proteins are restored to the chromatin-bound fraction by inhibition of protein kinase activity (Coverley et al., 2000). The MCM proteins, if lost from chromatin during S phase, cannot be replaced, as they are put in place by G1 processes involving the ORC, Cdc6/18 and Cdt1. The result of such loss during prolonged S phase arrest would be a cell that cannot complete S phase. Our evidence reveals that MCM3 and MCM4, two markers of the complex, are indeed partially depleted from the chromatin-bound fraction during prolonged S phase arrest.

Surprisingly, nuclei isolated from cells blocked for 60 hours in the presence of aphidicolin are as capable of initiating replication without added factors as are nuclei from S phase competent cells (30 hours arrest). In contrast, G1 nuclei (15 hours after release from serum starvation) used as controls in our experiments are incapable of incorporating biotin-dUTP. The incapacity of G1 nuclei to replicate is in accord with previous results which have shown that nuclei isolated from G1 cells do not have the capacity to replicate DNA unless incubated with S phase nuclei or extracts of S phase nuclei (Krude et al., 1997).

Following partial loss of MCM complex from the chromatin in prolonged drug treatment (Fig. 6), it is unlikely that in vitro replication progresses much beyond initiation. This result is nonetheless strikingly distinct from loss of replication capacity in intact cells. The in vitro assay of biotin-dUTP incorporation should not be interpreted as indicating the capacity for full replication in vitro. Positive nuclei would be generated with even partial replication. It has not been possible to quantitate the degree of replication in individual nuclei, but it is unlikely that, with the partial loss of MCM, complete replication competence is retained.

Since nuclei isolated from permanently arrested S phase cells are capable of initiating replication in vitro, we conclude that the replication machinery remains at least partially competent but that there is active suppression of replication in the intact cell following release from prolonged S phase arrest, presumably through checkpoint controls that read the inability of MCM-depleted chromatin to complete S phase. If such a checkpoint signal exists, it is likely to be readily diffused from nuclei during their isolation or inactivated by in vitro conditions, so that it does not function to suppress in vitro replication.

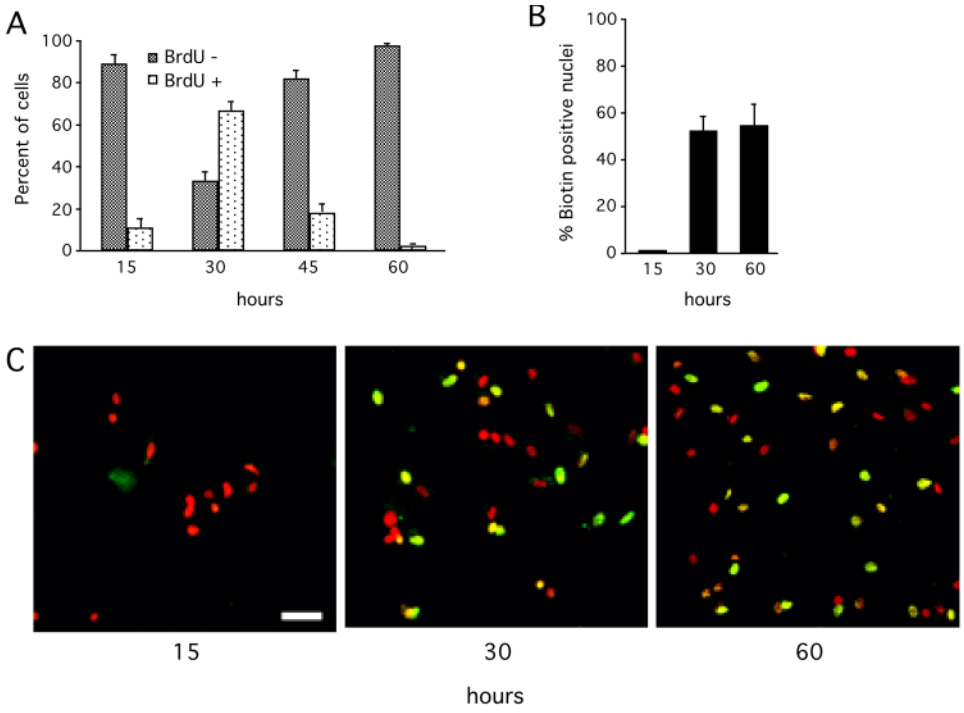

Fig. 7. DNA replication is suppressed in vivo following prolonged aphidicolin exposure, but extracted nuclei remain competent for replication. (A) In vivo analysis of DNA replication. Aphidicolin (10 μM)-treated REF-52 cells were released for 30 minutes in drug-free medium containing 10 μM BrdU and the percentages of replicating cells double-labeled for BrdU and for propidium iodide were determined by confocal immunofluorescence microscopy. An average of 1000 cells were counted in each of at least three samples at each time point. Values represent the percentages of BrdU-positive and -negative cells. Error bars represent the corresponding standard deviations. (B) Quantitative analysis of in vitro replicated nuclei derived from REF-52 cells and counts derived from microscopic imaging, as in part C. Following 15 hours of exposure to aphidicolin almost no nuclei (less than 1%) are positive. After 30 hours and 60 hours of exposure prior to harvest of nuclei, positive nuclei are 52% and 54% of the total, respectively. This experiment was replicated three times with similar results. An average of 450 nuclei were counted for each condition. (C) Visualization of in vitro DNA replication. REF-52 cells were released from G0 in the presence of aphidicolin (10 μM) and nuclei were harvested at the times indicated. Isolated nuclei were then subjected to in vitro DNA replication protocol as described in Materials and Methods. Fields of nuclei are shown following the replication assay, imaged for presence of biotin-16-dUTP and counterstained with propidium iodide. In the merged images green nuclei are biotin-16-dUTP positive and red nuclei are positive for only propidium iodide.

We note that HeLa cells exhibit behavior unlike that observed with the cells studied here. They undergo a partial replication on release from prolonged S phase arrest, followed by rapid apoptotic death (data not shown). HeLa are highly transformed and highly aneuploid. This result suggests that the alternative to maintenance of cells at the G1/S interface following partial loss of replicative capacity is rapid death following incomplete replication. We intend to address the possibility that transformed cells exhibiting chromosome instability may be uniquely sensitive to drugs that suppress S phase without damaging DNA. As aneuploidy and chromosomal instability (CIN) are characteristic of the great majority of human tumors (Cahill et al., 1998; Lengauer et al., 1997), and are linked to the progressive development of high-grade, invasive tumors (Giaretti, 1994; Rabinovitch et al., 1989; Sandberg, 1977), such a linkage between CIN status and increased sensitivity to replication inhibitors could be important.

The question arises whether S phase stasis occurs only in response to drug exposure, or if it could occur in response to physiological stimuli. The difference between our results in cells and in isolated nuclei suggests that a diffusible factor can specifically suppress replication in the absence of DNA damage in S phase. We therefore suggest that such a factor should have a role in suppressing replication as a normal physiological response. Further work will establish whether this is so. It is possible that the well-established cell cycle checkpoint triggered in response to incomplete DNA replication (Dasso and Newport, 1990) involves checkpoint machinery similar or identical to that described here in response to prolonged drug arrest. It is of interest that geminin, which we find persists in S phase arrested nuclei (Fig. 6), both suppresses replication (Wohlschlegel et al., 2000) and depletes XMCM7 from the chromatin-bound fraction (Tada et al., 2001) in an in vitro *Xenopus* extract system. It will be interesting to determine whether similar S phase stasis will occur in response to prolonged incomplete DNA replication created in the absence of drugs.

HU is used for treatment of myeloproliferative disorders such as essential thrombocythaemia (Green, 1999), but the mechanism of action that permits HU to be an effective treatment has not been clear. Our data suggest that transient suppression of replication may be augmented in the sensitive megakaryocyte cell population by permanent arrest of a portion of the cells exposed to HU. It remains to be seen whether megakaryocytes are especially sensitive to entry into stasis in the presence of HU. This possibility is interesting, as the S phase of differentiating megakaryocytes is distinct. Megakaryocytes undergo repeated endoreduplication without cell cleavage (Jackson, 1990), leading to multinucleation and polyploidy prior to the generation of platelets from the mature cell population. In this special case, the reloading of MCM proteins may be under controls that are distinct from those described above for normal cycling cells, and might thus be unusually sensitive to entry into S phase stasis.

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