Down-Regulation of DNA Replication in Extracts of Camptothecin-treated Cells: Activation of an S-phase Checkpoint?1

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Abstract

Extracts prepared from camptothecin (CPT)-treated cells have a reduced ability to support SV40 DNA replication in vitro. This reduction derives mainly from a reduction in the frequency of initiation events because DNA chain elongation remains practically unchanged. Mixing of extract from nontreated cells with small amounts of extract of CPT-treated cells indicates that the reduction in DNA replication is due to the synthesis/activation of a dominant inhibitor. The observed reduction in DNA replication activity cannot be attributed to inactivation of Topo I, the molecular target of camptothecin, because levels and activity of this protein remain unchanged in extracts of CPT-treated cells and addition of purified Topo I does not restore replication activity. Although replication protein A (RP-A) is phosphorylated in CPT-treated cells, reduced replication may not be caused by RP-A inactivation, because another loss of phosphorylation nor the addition of recombinant RP-A restore replication activity. We interpret these observations as biochemical evidence for the activation of a checkpoint in S phase and discuss the ramifications of this activation on the mechanism of CPT-induced cytotoxicity.

Introduction

CPT3 and its analogs are agents with a unique spectrum of antitumor activity mediated by a selective inhibition of eukaryotic DNA topoisomerase I (Topo I; for recent reviews, see Refs. 1–3). The cytotoxicity of these compounds is predominantly exerted during S phase and is associated with an inhibition of DNA replication. This inhibition is generally thought to be primarily the result of the passive collision of the advancing replication fork with the CPT-Topo I-DNA cleavable complex (4–8). Such collisions are expected to cause an inhibition in the elongation steps of DNA replication (9) and to kill cells by generating DNA dsbs (10, 11). However, not all effects of CPT can be explained by the collision model, and recently, evidence was presented that the sensitivity of cells to CPT may also be determined by their ability to activate checkpoints in S and G2 (12–14).

Because CPT toxicity is maximum during S phase (15–16), we hypothesized that if regulatory processes, such as those initiated in response to the activation of a checkpoint in S phase (15, 16), prevent replication on damaged/altered DNA template, they will operate as toxicity modifiers. Indeed, inhibition of DNA replication by aphidicolin reduces CPT toxicity (4, 9, 13). DNA replication inhibition as a result of the activation of regulatory processes is expected to have a similar outcome, with the significant difference that the protective mechanism will be under cellular control. Information on the molecular determinants of this regulation may offer new ways of intervention for drug sensitization. Therefore, we sought biochemical evidence for the operation of regulatory processes inhibiting DNA replication in human cells exposed to CPT. We examined the ability of extracts prepared from CPT-treated cells to support DNA replication using the SV40 based in vitro replication assay (17–19). In this assay, replication of plasmids carrying the SV40 origin of DNA replication is achieved with extracts prepared from human cells. All proteins required for replication, except for the SV40 TAg, are of cellular origin. The assay allows the evaluation of extracts prepared from CPT-treated cells for the presence of regulatory factors, as well as for the inactivation of essential replication factors. Because the plasmid DNA substrate is never exposed to CPT, complications otherwise arising when damaged DNA is used as substrate (as with experiments carried out in vivo) are eliminated. We have previously validated this assay in experiments designed to characterize the regulation of DNA replication in cells exposed to ionizing radiation (20, 21).

Materials and Methods

Cell Culture and Drug Treatment. HeLa cells were grown in spinner flasks seeded at 0.5–1 × 10^6 cells/ml at volumes that ranged from 0.5 to 10 liters for 3–4 days at 37°C in Joklik-modified MEM (S-MEM) supplemented with 5% iron-supplemented calf serum (Sigma Chemical Co., St. Louis, MO). CPT sodium salt (NCS100800, obtained from the National Cancer Institute) was added to mid-logarithmic phase cultures (4–6 × 10^6 cells/ml) at 37°C for 3 h. Cells were processed for extract preparation immediately after completion of treatment.

Cell Extract Preparation and in Vitro Assay of DNA Replication. The methods used for extract and TAg preparation, as well for the assembly of the in vitro reactions, have been described previously (20, 21). Extracts (S-100) and TAg were stored in small aliquots at −70°C. The plasmid DNA, pSV0ΔEFP, carrying the minimal origin of SV40 DNA replication, was used as template in the replication reactions (kindly provided by Dr. J. Hurwitz, Memorial Sloan-Kettering Cancer Center, New York, NY). The reaction mixture was incubated at 37°C for 1 h. Reactions were terminated by adjusting the mixture to 20 mM EDTA. Activity present in acid insoluble material was determined as described (19). Replication products were analyzed by agarose gel electrophoresis.

DNA Synthesis on a Primed M13 ssDNA. The reaction mixture (25 µl) contained 30 mM Hepes (pH 7.8), 7 mM MgCl₂, 0.5 mM DTT, all four dNTPs (each at 50 µM), [α-^32P]dCTP (2000 cpm/µmol), 100 ng of M13 ssDNA (M13mp18) primed with a 3-fold molar excess of a unique 17-base primer (primer 1211; New England BioLabs, Beverly, MA), and 125 µg of S-100 extract. The reaction was incubated at 37°C for 1 h, and ^32P incorporation into DNA was determined by liquid scintillation counting as described above. To analyze replication products, DNA was extracted with phenol/chloroform (1:1), precipitated with ethanol, and dissolved in 20 µl of 10 mM Tris-1 mM EDTA (TE). The sample was subjected to electrophoresis in an alkaline agarose gel (1%) in 30 mM NaOH-1 mM EDTA at 1.5 V/cm for 12 h. Before being dried for autoradiography, the gel was fixed in 10% methanol-10% acetic acid. Other details were as described previously (22).
Western Blotting. Cells were lysed in SDS-PAGE loading buffer and extracts were prepared as described above. Proteins were separated by SDS-PAGE on a 12% gel and transferred to a polyvinylidene difluoride membrane. Western blot analysis was performed using enhanced chemiluminescence according to the manufacturer (Amersham Corp.). Anti-RP-A antibody (p34-20; generously provided as ascites fluid by Dr. Bruce Stillman, Cold Spring Harbor Laboratory, NY) was diluted 1:4000. Anti-Topo I antibody (generously provided as cell supernatant by Dr. Y.C. Cheng, Yale University, New Haven, CT) was diluted 1:200.

RP-A Purification. The methodology used was essentially as described (23). Briefly, bacteria [strain BL21 (DE3)] were transformed with the expression vector pld-hRP-A containing the cDNA sequences for 70,000, 32,000, and 40,000 of human RP-A (Ref. 20); a gift from Wold, University of Iowa College of Medicine, Iowa City, IA), and a 1-liter culture was grown to an A600 nm of 0.6. At this point, the culture was induced with 0.2 mM isopropyl thiogalactoside and allowed to grow for 2 h at 37°C. Cells were collected by centrifugation and lysed by sonication in 5 ml of HI buffer (30 mM HEPES, pH 7.8, 1 mM DTT, 0.25 mM EDTA, 0.25% (w/v) insolubilized; and 0.01% (w/v) Nonidet P-40) supplemented with 1 mM phenylmethylsulfonyl fluoride. The lysate was centrifuged, and the sample was loaded on an Affi-Gel blue column equilibrated with HI buffer containing 50 mM KCl. The column was washed with 50 ml each of 50 mM KCl, 0.8 mM KCl, and 0.5 mM NaSCN. RP-A eluted with 1.5 mM NaSCN. Peak fractions were loaded on a hydroxyapatite column equilibrated with HI buffer. Protein was eluted with four column volumes of HI buffer containing 80 mM K2PO4. The resulting peak fractions were applied to a Mono-Q column equilibrated with HI buffer containing 100 mM KCl. The column was developed with a 10-ml linear KCl gradient (100-400 mM KCl). RP-A elutes with 300 mM KCl and is homogeneous by SDS-PAGE followed by silver staining.

Topo I Purification and Activity Measurements. Topo I was purified from HeLa cell nuclei according to the method of Liu and Miller (24). Briefly, HeLa cell nuclei were lysed, and DNA was precipitated with polyethylene glycol. The resulting supernatant was loaded on a hydroxyapatite column equilibrated with HI buffer containing 70 mM KCl. The column was washed with 50 ml each of 50 mM KCl, 0.8 mM KCl, and 0.5 mM NaSCN. RP-A eluted with 1.5 mM NaSCN. Peak fractions were loaded on a hydroxyapatite column equilibrated with HI buffer. Protein was eluted with four column volumes of HI buffer containing 80 mM K2PO4. The resulting peak fractions were applied to a Mono-Q column equilibrated with HI buffer containing 100 mM KCl. The column was developed with a 10-ml linear KCl gradient (100-400 mM KCl). RP-A elutes with 300 mM KCl and is homogeneous by SDS-PAGE followed by silver staining.

Topo I activity during purification and for the experiments described here was measured by the relaxation of superhelical plasmid DNA (pUC18, or pSV01AEP Ref. 24). The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 120 mM KCl, 10 mM MgCl2, 0.5 mM DTT, 0.5 mM EDTA, 30 mg/ml BSA, 20 mM/μl plasmid DNA, and various amounts of enzyme. The reactions were incubated at 37°C for 30 min and were stopped by the addition of 5 μl of 5% SDS, 25% Ficoll-400, containing 1 mM phenylmethylsulfonyl fluoride. The column was developed with a 0.2-0.7 M potassium phosphate gradient, and the fractions containing Topo I activity were pooled and loaded on a phosphocelululose (P-11) column. Topo I was eluted with 0.2-0.7 M potassium phosphate linear gradient, and fractions containing activity were pooled and dialyzed. Finally, active fractions were loaded on ssDNA cellulose. Topo I activity eluted in the 0.4 M KCl wash and was homogeneous by SDS-PAGE and silver staining.

Topo I activity in vivo was measured by the relaxation of superhelical plasmid DNA (pUC18, or pSV01AEP Ref. 24). The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 120 mM KCl, 10 mM MgCl2, 0.5 mM DTT, 0.5 mM EDTA, 30 mg/ml BSA, 20 mM/μl plasmid DNA, and various amounts of enzyme. The reactions were incubated at 37°C for 30 min and were stopped by the addition of 5 μl of 5% SDS, 25% Ficoll-400, containing 0.25 mg/ml bromophenol blue. Samples were electrophoresed for 2 h in 1% agarose with 0.5X TBE (45 mM Tris pH 8.2, 45 mM boric acid, and 1 mM EDTA) at 2 V/cm and visualized by staining with ethidium bromide. One unit is defined as the amount of enzyme required to relax 0.5 μg of plasmid in 30 min at 37°C.

Measurement of DNA Replication in Intact Cells. Aliquots of cells (3.0 ml) from the same spinner cultures used to prepare S-100 cytoplasmic extracts were supplemented with 3 μCi of [3H]thymidine. Samples were incubated at 37°C for 15 min in an atmosphere of 5% CO2 and 95% air. Cells were then collected and loaded on GF/A glass fiber filters, washed with 10% trichloroacetic acid and then washed with deionized water. [3H]Radioactivity was measured using liquid scintillation counting.

PFGE. Asymmetric field inversion gel electrophoresis, a PFGE method, was carried out in 0.5% agarose (FMC Bioproducts) in 0.5X TBE at 10°C for 40 h, by applying cycles of 1.25 V/cm for 900 s in the direction of DNA migration and 5.0 V/cm for 75 s in the reverse direction. Cells were treated with CPT in dishes and embedded, after trypsinization, in agarose, where they were lysed before electrophoresis. Quantification and analysis for DNA dsbs present were carried out in a PhosphorImager (Molecular Dynamics), or by scintillation counting. dsbs were quantified by calculating the fraction of activity released from the well into the lane, which equals the ratio of the radioactivity signal in the lane versus that of the entire sample (well plus lane) in CPT-treated and nontreated samples. Details of the assay have been published (25).

Results

Reduced DNA Replication Activity in Extracts of CPT-treated Cells. Treatment for 3 h with 0.5 μM CPT reduces incorporation of [3H]thymidine into DNA of HeLa cells to 18% of untreated controls, indicating a strong inhibition of DNA replication (Fig. 1A, first column). This inhibition may be due to damage that alters the substrate properties of the DNA during replication. It is known that a collision of the advancing replication fork with the cleavable complex will inhibit DNA replication and will lead to the formation of DNA dsbs (see "Introduction"). Indeed, exposure for 3 h of HeLa cells to CPT leads to a dose-dependent formation of dsbs that can be assayed immediately thereafter by PFGE (Fig. 2). A comparison between results of cells exposed to CPT and results of cells exposed to X-rays (Fig. 2), suggests that cells exposed for 3 h to 4 μM CPT have in their

Fig. 1. A, DNA synthesis as measured by incorporation of [3H]thymidine in exponentially growing HeLa cells (in vitro) incubated for 3 h with 0.5 μM CPT (first column); results are normalized to the levels of DNA synthesis observed in nontreated cells. DNA synthesis in vitro, using the SV40-based in vitro replication assay and extracts (125 μg) prepared from HeLa cells exposed to 0.5 μM CPT for 3 h (second column); results are normalized to the levels of DNA synthesis observed with extracts of nontreated cells. In vitro DNA replication using extracts of nontreated cells (125 μg) in the presence of 0.5 (third column) and 300 μM (fourth column) CPT. B, Agarose gel electrophoresis and radioautography of the products of DNA replication reactions carried out in vitro in the presence of extracts from nontreated or CPT-treated cells as described in A. Shown are also results of a reaction assembled by mixing 100 μg of extract of nontreated cells with 10 μg of extract of CPT-treated cells. C, DNA chain elongation catalyzed by extracts of nontreated or CPT-treated cells using primed M13 DNA as a substrate. Columns, mean from four replicate experiments; bars, SE.
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DNA a number of dsbs approximately equivalent to that produced by an acute dose of 6-Gy X-rays. Assuming a linear correlation between fraction of activity released and dose, we calculate that cells exposed to 0.5 μM CPT will carry a load of dsbs equivalent to that of 0.8-Gy X-rays. It is unlikely that this load of dsbs can, by itself, cause the level of replication inhibition observed in Fig. 1A (~80%), as 10-Gy X-rays cause a reduction in DNA synthesis by only 10–15% (26, 27).

We inquired, therefore, whether effects other than damage in the DNA substrate contribute to the inhibition of DNA replication observed in intact cells. In particular, we were interested in examining whether inactivation of key replication enzymes or the activation of a checkpoint in S phase contributed to the inhibition. As a first step towards this goal, we compared the replication activity of extracts prepared from CPT-treated cells to that of untreated controls using the SV40-based in vitro replication assay. We reasoned that this assay, by allowing a separation of the substrate DNA from the enzymatic machinery (it uses undamaged plasmid DNA as a substrate, rather than the damaged cellular DNA of in vivo experiments), offers a unique way to investigate enzyme inactivation/depletion and regulatory factor production as contributors to the observed down-regulation of DNA replication. The results in Fig. 1A (second column) indicate that extracts of cells exposed for 3 h to 0.5 μM CPT show a strong reduction in their ability to support DNA replication in vitro. In fact, the level of inhibition observed in cell extracts tested in vitro (80%) is comparable to the inhibition observed in intact cells tested in vivo (82%), suggesting that effects, in addition to damaged DNA, contribute to the DNA replication arrest observed in cells exposed to CPT. There was no evidence for apoptosis in cells treated with CPT under the conditions used to prepare the extracts.

It is not possible to attribute part of the inhibition observed in in vitro replication reactions assembled with extract of CPT-treated cells to the presence of free CPT because unbound drug is removed during extract preparation in the final dialysis step. To evaluate the direct effects of CPT on DNA replication, we tested the effect of 0.5 and 300 μM CPT on DNA replication in reactions assembled with extract from nontreated cells. The drug was added directly to the reaction mixture and DNA synthesis was measured. The results in Fig. 1A (third and four columns) confirm previous observations (7) and suggest that CPT has only a small direct effect on DNA replication, even at concentrations 200-fold higher than those administered to cells.

Topo I Levels and Activity Are Unchanged in Extracts of CPT-treated Cells. The reduction in replication activity of extracts prepared from CPT-treated cells may be due to an inhibition/depletion of key replication factors. Because CPT is known to specifically interact with DNA-bound Topo I to trap the cleavable complex, we inquired whether this effect causes an inactivation or depletion of the enzyme in the extract. Western blots for Topo I in extracts of nontreated or CPT-treated cells do not show a reduction in enzyme levels (Fig. 3A). In addition, activity measurements, using as an endpoint the relaxation of supercoiled plasmid DNA, demonstrate no change in the overall activity in extracts from CPT-treated cells (Fig. 3 B and C). Shown in the figure for comparison are also results obtained with extract of cells exposed to 50-Gy X-rays.

As a final confirmation that inactivation of Topo I may not underlie the reduced replication activity of extracts of CPT-treated cells, we added purified Topo I in reactions assembled with extracts of nontreated or CPT-treated cells. The results show (Fig. 3D) that addition of purified Topo I does not affect the replication activity of extracts of either nontreated or CPT-treated cells.

Evidence for Factors in Extracts of CPT-treated Cells that Inhibit DNA Replication. We inquired whether extracts of CPT-treated cells contain factors that inhibit DNA replication. For this purpose, replicate reactions were assembled with 100 μg of extract of nontreated cells, and the indicated amount of extract from CPT-treated or nontreated cells was added. Reactions were incubated at 37°C for 1 h to measure DNA synthesis. The results obtained are shown in Fig. 4A. Addition of the extract of nontreated cells increased replication activity. However, addition of extract from CPT-treated cells rapidly reduced replication activity from 23 to about 6 pmol. These results suggest the presence of a replication inhibitor in the extract of CPT-treated cells. The inhibitor must be a high molecular weight molecule because it is retained during dialysis in a membrane with a cutoff of $M_w$ 14,000.

The Reduction in DNA Replication in Extracts of CPT-treated Cells Reflects Mainly Inhibition of Replicon Initiation. In an effort to examine further the mechanism of inhibition, we sought information regarding the step of DNA replication preferentially inhibited. For this purpose, we examined the size distribution of newly formed DNA in the in vitro reactions. Fig. 1B shows an analysis of the replication products by gel electrophoresis followed by radioautography using a PhosphorImager. The size distribution of newly produced (radioactivity-labeled) DNAs is similar in reactions assembled with extracts of nontreated and CPT-treated cells, despite the fact that much less DNA is synthesized in the latter reaction. Similar results were also obtained in reactions assembled with extract of nontreated cells in the presence of small amounts of extract from CPT-treated cells. These observations are compatible with an inhibition of the initiation events of DNA replication in extracts of CPT-treated cells. Inhibition of DNA chain elongation would have produced a more diffused pattern of products and would have increased the presence of small DNA molecules, neither of which was actually observed.

We confirmed that DNA chain elongation is not affected in extracts of CPT-treated cells by measuring DNA chain elongation using primed M13 ssDNA and extracts of nontreated and CPT-treated cells. Cell extracts (S-100) contained all factors required for elongating a primer on a M13 substrate. No reduction was observed in the ability of extracts of CPT-treated cells to support DNA chain elongation, and actually a slight increase in this ability is reproducibly found (Fig. 1C). Also, the size distribution of the synthesized DNA was similar in reactions assembled with extract from nontreated and CPT-treated cells (results not shown). In a separate experiment, we examined the effect of CPT on DNA chain elongation using the above described assay and extracts of nontreated cells. There was no effect of CPT on...
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Fig. 3. A, Western blot of Topo I in extracts of nontreated and CPT-treated cells. Protein equivalent to 2 × 10⁵ cells was loaded per well. B, Topo I activity as measured by the relaxation after various periods of incubation of supercoiled plasmid (pSV01E) in extracts of nontreated and CPT-treated cells (50 ng). Shown for comparison are also results obtained from extracts of cells exposed to 50-Gy X-rays. C, quantitation of the results shown in B using a FluorImager. Pointed is the fraction of plasmid DNA remaining supercoiled as a function of the incubation time. D, in vitro DNA replication with extracts of nontreated and CPT-treated cells in the presence of various amounts of purified Topo I.

Discussion

If fork collisions with cleavable complexes, as predicted by the "fork collision model," were the only mechanism by which CPT inhibited DNA replication, damage/alterations in the substrate DNA would be the main cause of the inhibition. Thus, in an experiment designed to separate the damaged DNA substrate from the enzymatic machinery, as is possible using the SV40 in vitro replication assay, one would expect levels of replication of plasmid DNA comparable to those observed with extracts of non-treated cells. However, the results presented in Fig. 1A suggest that extracts prepared from CPT-treated cells are significantly impaired in their ability to replicate plasmid DNA. Indeed, the level of replication inhibition observed in vivo is very similar to that observed in vitro, suggesting that the determinants of inhibition are the same under both conditions.

We hypothesize, therefore, that the reduced DNA replication activity of extracts prepared from CPT-treated cells is due to the formation or activation of a regulatory molecule(s) that inhibits DNA replication in vivo and in vitro. The activation of such a regulator of DNA replication would reflect the activation of a surveillance mechanism in response to DNA replication inhibition or DNA damage production and will be equivalent to the activation of a checkpoint in S (14-16). Several observations suggest that the effects of CPT on DNA replication and cytotoxicity may be determined by effects in addition to those directly deriving from DNA damage. First, the amount of DNA damage (dsbS in our experiments) present in CPT-treated cells is relatively low and cannot explain the level of DNA replication inhibition observed (Figs. 1A and 2). Second, DNA replication per se is rather resistant to CPT exposure, as indicated by experiments in which 300 μM CPT was added to reactions measuring replication in vitro (Ref. 7; results in Fig. 1A). This would suggest that the inhibition observed in vivo at CPT concentrations as low as 0.1 μM may not reflect solely a direct inhibition of the replication machinery, but rather the combination of a direct DNA damage-related inhibition and...
and an indirect inhibition induced by the activation of a checkpoint. The small direct effect of CPT on DNA replication in vitro and the strong inhibition observed in vivo and in extracts prepared from CPT-treated cells would actually suggest that the indirect mechanism dominates the response. Third, inhibition of DNA replication in CPT-treated cells usually persists for a long time after drug removal and does not correlate with the presence of DNA damage or the trapping of Topo I-DNA cleavable complexes (13), which again suggests the activation of checkpoints.

Several lines of investigation suggest the activation of checkpoints in cells exposed to CPT. Cells treated with CPT are blocked in G2, an indication of the activation of a checkpoint in this phase of the cell cycle (1–3, 12). Recently, evidence has been presented that CPT cytotoxicity may be affected by the ability of cells to activate checkpoints in S and G2 (13). Given the specificity of CPT action on S-phase cells, it seems reasonable that these cells will mount an active response after such treatment to minimize its adverse effects. Such a response may be initiated by the DNA damage produced and may have important consequences in overall cytotoxicity (36).

Our results suggest that DNA replication is regulated in CPT-treated cells by a dominant factor that specifically inhibits the initiation steps of DNA replication. This mode of inhibition is different from that associated with the direct effect of CPT that is thought to be limited to chain elongation (7). It is particularly interesting in this regard that chain elongation activity is not inhibited in extracts of CPT-treated cells (Fig. 1C). The nature of the DNA replication inhibitor is presently unknown but it seems unlikely to be RP-A because inactivation of this replication factor would not be expected to be dominant in mixing experiments. More importantly, recombinant RP-A fails to restore DNA replication activity (Fig. 4C). We observed phosphorylation of RP-A p34 in response to CPT treatment (Fig. 4B), suggesting the operation of regulatory processes on the protein. It has been reported that throughout the cell cycle, p34\textsuperscript{cdc2} kinase, in association with cyclin A or B, phosphorylates RP-A p34 at Ser-23 and Ser-29 (37). Other reports indicate that RP-A p34 is phosphorylated in two steps, first by cdk-cyclin A and then by DNA-PK, and that DNA-PK prevents this phosphorylation in cyclin A-activated extracts of G1 cells (38). DNA-PK has been purified as the kinase responsible for RP-A p34 phosphorylation during in vitro replication (39). However, no direct association between RP-A p34 phosphorylation and DNA replication activity could be established (38–41). Ionizing radiation-induced phosphorylation of RP-A was found to be lacking in scid cells known to be deficient in DNA-PK (42), but these results are not confirmed by recent reports (43). Thus, the RP-A kinase responding to DNA damage remains to be identified. The role of RP-A p34 phosphorylation in RP-A activity...
remains uncertain, and our results suggest that RP-A may not be contributing to the replication inhibition observed in extracts of CPT-treated cells. However, indirect effects of RP-A on the regulation of DNA replication cannot be excluded.

In summary, we have presented evidence for factors inhibiting DNA replication in extracts of CPT-treated cells. We showed that these factors act in a dominant fashion and that they specifically inhibit replicon initiation; their effects are distinct from the direct effect of CPT on chain elongation. We propose that these factors are the effectors of an S-phase checkpoint and that they are important determinants of drug toxicity. The focus of future research will be the identification and characterization of these factors.

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