

## DNA Lesion-specific Co-localization of the Mre11/Rad50/Nbs1 (MRN) Complex and Replication Protein A (RPA) to Repair Foci\*

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**The DNA damage response, triggered by DNA replication stress or DNA damage, involves the activation of DNA repair and cell cycle regulatory proteins including the MRN (Mre11, Rad50, and Nbs1) complex and replication protein A (RPA). The induction of replication stress by hydroxyurea (HU) or DNA damage by camptothecin (CAMPT), etoposide (ETOP), or mitomycin C (MMC) led to the formation of nuclear foci containing phosphorylated Nbs1. HU and CAMPT treatment also led to the formation of RPA foci that co-localized with phospho-Nbs1 foci. After ETOP treatment, phospho-Nbs1 and RPA foci were detected but not within the same cell. MMC treatment resulted in phospho-Nbs1 foci formation in the absence of RPA foci. Consistent with the presence or absence of RPA foci, RPA hyperphosphorylation was present following HU, CAMPT, and ETOP treatment but absent following MMC treatment. The lack of co-localization of phospho-Nbs1 and RPA foci may be due to relatively shorter stretches of single-stranded DNA generated following ETOP and MMC treatment. These data suggest that, even though the MRN complex and RPA can interact, their interaction may be limited to responses to specific types of lesions, particularly those that have longer stretches of single-stranded DNA. In addition, the consistent formation of phospho-Nbs1 foci in all of the treatment groups suggests that the MRN complex may play a more universal role in the recognition and response to DNA lesions of all types, whereas the role of RPA may be limited to certain subsets of lesions.**

Both replicative stress and DNA damage initiate cellular processes collectively termed the DNA damage response. These processes include activation of appropriate DNA repair mechanisms, delay of the cell cycle in order to allow sufficient time for repair, and in some cases apoptosis (1, 2). One hallmark of the DNA damage response is the aggregation of multiprotein complexes into foci or repair centers. The composition of the foci depends upon the nature of the DNA lesion and is temporally dynamic, changing as the damage is first recognized, processed, and then repaired (3, 4). The assembly of foci appears to be largely governed by a network of protein-protein interac-

tions rather than DNA-protein interactions (3, 4), providing an explanation for the dynamic composition of the foci. Although different types of DNA damage activate specific repair pathways and therefore specific proteins, there are some protein factors that respond to multiple types of lesions (3–6). These global response proteins are organized in a distinct spatiotemporal fashion depending upon the nature of the DNA lesion (3, 4). Two protein complexes that are intimately involved in the DNA damage response to multiple types of lesions are the Mre11/Rad50/Nbs1 (MRN)<sup>1</sup> complex and replication protein A (RPA).

The MRN complex is best known for its role in DNA double-strand break (DSB) repair (7), but more recent evidence has expanded this role to include participation in DNA replication, cell cycle checkpoints, telomere maintenance, signaling/sensing of DNA damage, and response to stalled replication forks (3, 8–15). The MRN complex forms foci at sites of DNA replication, DNA damage, and DNA repair (3, 15–20). Both Mre11 and Nbs1 become phosphorylated following treatment of cells with genotoxic agents (21, 22), and Mre11 is phosphorylated in a DNA replication-dependent manner (10). The functional significance of these phosphorylation events is not entirely known, but Nbs1 phosphorylation affects cell cycle checkpoint activation (23–25) and Mre11 phosphorylation may increase its nuclease activity (10).

RPA, a three-subunit protein (p70, p34, and p14, named for their apparent molecular weights), is the major single-stranded DNA (ssDNA)-binding protein in mammalian cells (replication factor A in yeast). RPA is an essential protein that plays a crucial role in DNA replication, repair, and recombination (26). RPA coats the ssDNA found at sites of DNA replication (27–31), DNA damage, and DNA repair (3, 19, 32–35). It has been postulated that this RPA-DNA intermediate is a common signal necessary to activate the DNA damage response (36, 37). The p34 subunit of RPA is hyperphosphorylated in response to replicative stress and DNA damage (15, 38–40). This hyperphosphorylation is postulated to function as a “switch” to direct RPA activity from DNA replication to DNA repair (41–43).

We have previously shown that the MRN complex and RPA co-localize to stalled replication forks where they interact following replicative stress induced by hydroxyurea (HU) or UV (15). We wanted to determine whether the interaction of the MRN complex with RPA was specific to replicative stress responses or whether other types of DNA damage also stimulate the interaction. To achieve this goal, we used topoisomerase inhibitors and cross-linking agents. Whereas HU leads to rep-

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<sup>1</sup> The abbreviations used are: MRN, Mre11/Rad50/Nbs1; RPA, replication protein A; HU, hydroxyurea; ETOP, etoposide; CAMPT, camptothecin; MMC, mitomycin C; DSBs, double-strand breaks; SSBs, single-strand breaks; ssDNA, single-stranded DNA; PBS, phosphate-buffered saline.

lication fork stalling due to depletion of deoxyribonucleotide substrates (44, 45), camptothecin (CAMPT), a topoisomerase I inhibitor (46, 47), leads to single-stranded DNA breaks. Both HU and CAMPT have the potential to ultimately induce DNA DSBs, HU through the collapse of stalled replication forks (48–50) and CAMPT through the replication of nicked template DNA (47, 51, 52). DSBs also can be caused by etoposide (ETOP), a potent inhibitor of topoisomerase II (46, 53–55), and mitomycin C (MMC), a DNA cross-linking agent (56, 57), that generates DNA DSBs through the repair process of the cross-links (58–60).

Here we demonstrate that co-localization of the MRN complex and RPA in nuclear foci differs depending on the damaging agent used. HU and CAMPT treatment led to phospho-Nbs1 foci that co-localized with RPA foci. In contrast, ETOP treatment induced both phospho-Nbs1 and RPA foci but not within the same cell. Treatment with MMC induced phospho-Nbs1 foci but did not induce any RPA foci formation until the latest time point investigated. These localization differences suggest that, although the DNA damage response is activated (evidenced by Nbs1 phosphorylation) in response to HU, CAMPT, ETOP, and MMC, the interaction of the MRN complex and RPA at the level of co-localization into repair foci is limited to responses to HU and CAMPT.

#### MATERIALS AND METHODS

**Cell Lines and Treatments**—HeLa cells were obtained from American Type Cell Culture (Manassas, VA) and maintained at 37 °C and 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 1% penicillin-streptomycin (Invitrogen). For HU (Sigma) treatment, asynchronous cells were incubated in growth medium containing 4 mM HU for 1–24 h before harvesting. For CAMPT (Sigma) treatment, the drug was added directly to the cell medium at a final concentration of 1 μM for 1–6 h before cell harvest. For ETOP (Sigma) treatment, cells were incubated in growth medium containing 25 μM ETOP for 1–6 h before harvesting, whereas for MMC (Sigma) treatment, the drug was added to the media at a final concentration of 1 μg/ml for 1–6 h before cell harvest.

**Immunofluorescent Detection of Foci**—Cells were grown on 12-mm coverslips (BD Biosciences Labware, Bedford, MA) for 48 h prior to treatment. Cells were treated with 4 mM HU for 1–24 h, 1 μM CAMPT for 1–6 h, 25 μM etoposide for 1–6 h, or 1 μg/ml MMC for 1–6 h. After treatment, cells were fixed with phosphate-buffered saline (PBS) containing 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), permeabilized with PBS containing 0.5% Triton X-100 (Sigma), and then blocked in PBS containing 15% fetal bovine serum. After incubation in primary antibody solutions overnight at 4 °C, cells were washed with PBS, incubated in secondary antibody solutions at room temperature, washed with PBS, and then placed on slides using gel-mounting medium (Biomedica, Foster City, CA) containing 4',6-diamidino-2-phenylindole as a counterstain. Primary antibody dilutions used were as follows: anti-RPA (1:1500) (NeoMarkers, Fremont, CA) and anti-Nbs1-S<sup>P</sup>343 (1:1500) (Novus Biological). Secondary antibody dilutions used were as follows: anti-rabbit Alexa Fluor 488 (1:250) and anti-mouse Alexa Fluor 594 (1:250) (Molecular Probes, Eugene, OR). The percentages of cells containing foci were determined using a Nikon inverted fluorescent microscope, and images of the foci were captured using a Zeiss Axiovert 100M equipped with a Zeiss LSM 510 laser-scanning confocal microscope. Images were processed using Adobe Photoshop 7.0 (Adobe, San Jose, CA). For each experiment, at least 100 cells were analyzed and experiments were repeated three times.

**Western Immunoblots**—Cell lysates were separated on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were probed using anti-RPA-p34 (1:5000) and horseradish peroxidase-linked anti-mouse antibodies (Amersham Biosciences, Buckinghamshire, England; 1:3,000). Bound antibodies were visualized using chemiluminescent detection.

**Comet Assays**—Neutral comet assays were performed as described previously (61, 62) with slight modifications. 12,000–15,000 cells from treated cultures were suspended in low-melting point agarose in PBS (Invitrogen, final concentration of 0.75%) and layered on microscope slides. The slides then were placed in neutral lysis buffer (1 mM Tris-HCl, pH 7.4, 150 mM NaCl, 4 mM EDTA, and 18 mM *N*-laurylsarcosine)

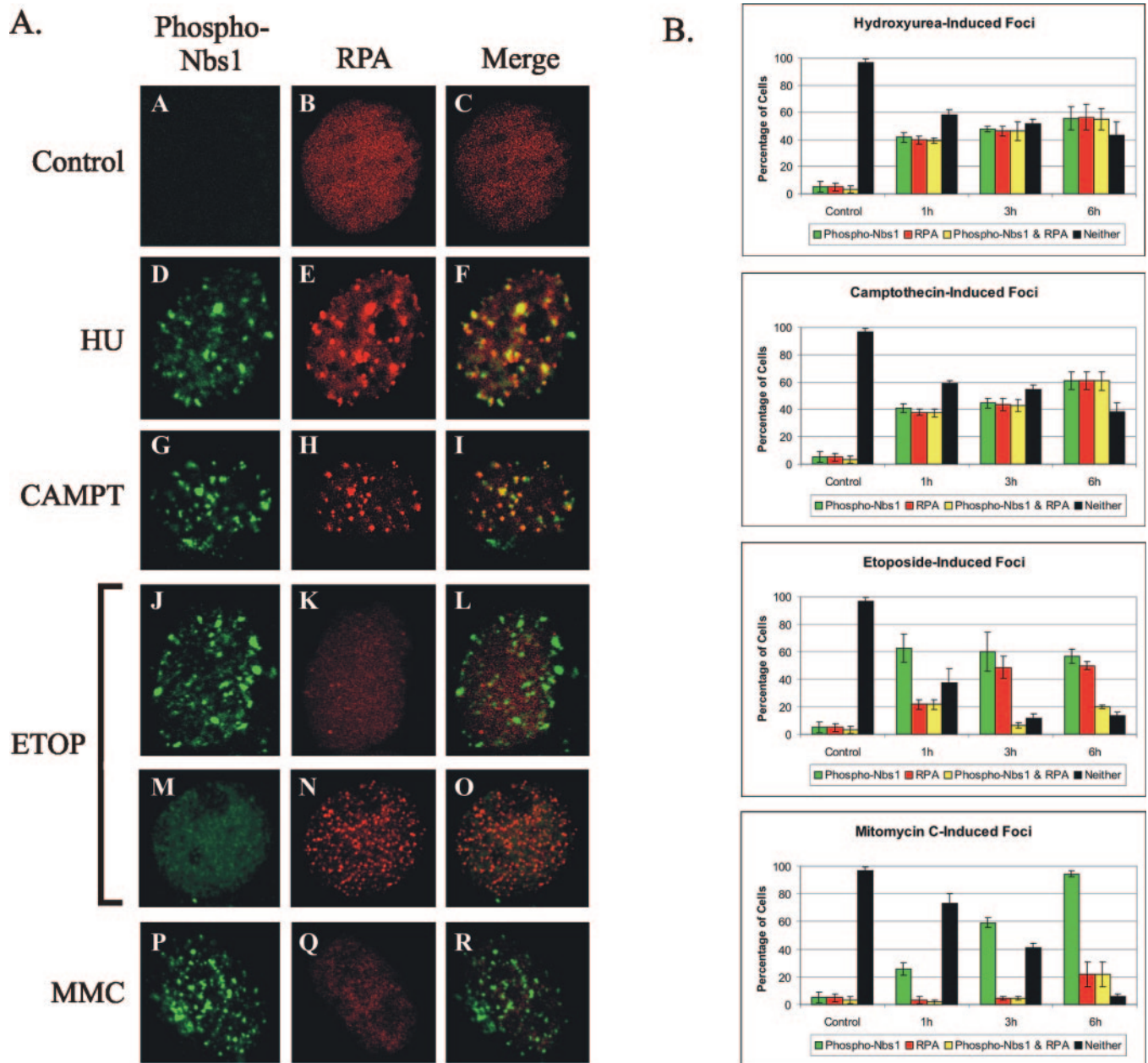
for 3 min and then placed into deionized H<sub>2</sub>O for 10 min. Electrophoresis was done at 20 V, 200 mA for 10 min in electrophoresis buffer (230 mM Tris, 180 mM boric acid, and 0.2 mM EDTA). Following electrophoresis, cells were immersed in a propidium iodide solution (2.5 μg/ml, Sigma) for 20 min and rinsed in deionized H<sub>2</sub>O and coverslips were placed on the gels. Slides were analyzed using a Nikon inverted fluorescent microscope with attached CCD camera. Images were saved as Bitmap files, and tail moments were determined using CometScore™ Freeware from TriTek Corp. (Sumerduck, VA).

#### RESULTS

**HU and CAMPT Induce Co-localization of Phospho-Nbs1 and RPA Foci**—The MRN complex and RPA both form foci at stalled replication forks and sites of DNA damage (3, 15–20, 32–34). We have previously reported that Mre11 and RPA foci co-localize following replicative stress (15), and consistent with that report, we observed co-localization of phospho-Nbs1 and RPA foci following HU treatment (Fig. 1A, panels D–F). In a similar fashion, cells treated with CAMPT, a topoisomerase I inhibitor (46, 47), also formed phospho-Nbs1 and RPA foci that co-localized (Fig. 1A, panels G–I). To determine the extent of foci formation, we calculated the percentage of cells containing phospho-Nbs1 foci, RPA foci, or both. Although not addressing co-localization, the percent of cells expressing foci provided information on the ability of the MRN complex and RPA to form foci independently of each other. The percentage of cells that contained phospho-Nbs1, RPA, or both phospho-Nbs1 and RPA foci showed a marked similarity between HU and CAMPT treatment. The percentage of cells containing phospho-Nbs1 foci increased from ~40% at 1 h to 60% at 6 h. Notably, the cells that contained phospho-Nbs1 foci also contained RPA foci and the reverse was also true. (Fig. 1B). With these agents, the phospho-Nbs1 and RPA foci co-localized, implying that the MRN complex and RPA responded jointly at both stalled replication forks and DNA single-strand breaks (SSBs).

**ETOP and MMC Induce Phospho-Nbs1 and RPA Foci That Do Not Co-localize**—To determine the extent of phospho-Nbs1 and RPA foci formation and to determine whether these proteins co-localize at other DNA lesions, we investigated two additional agents that damage DNA by different mechanisms: ETOP, a topoisomerase II inhibitor (46, 53), and MMC, a DNA cross-linking agent (56, 57). In contrast to the HU- and CAMPT-induced foci, ETOP and MMC treatment did not lead to phospho-Nbs1 and RPA foci co-localization (Fig. 1A, panels L, O, and R). Following treatment with ETOP, the majority of cells contained either phospho-Nbs1 or RPA foci but not both (Fig. 1, A, panels J–O, and B). After 1 h of ETOP treatment, ~60% cells had phospho-Nbs1 foci and ~20% had both phospho-Nbs1 and RPA foci. At later time points, an equivalent percentage of cells (~50–55%) demonstrated either phospho-Nbs1 or RPA predominant foci. However, the percentage of cells that contained both phospho-Nbs1 and RPA foci dropped to 5% at 3 h and had risen to ~20% by 6 h (Fig. 1B).

Treatment with MMC led to an increase in the percentage of cells containing phospho-Nbs1 foci from ~25% after 1 h of treatment to 95% after 6 h of treatment (Fig. 1B). Notably, RPA foci were not seen until the 6-h time point where only ~20% cells, which contained phospho-Nbs1 foci, also contained RPA foci (Fig. 1B). Coupled with the ETOP data, this difference in co-localization suggested that the response of the MRN complex and RPA was not equivalent for all types of lesions. Whereas the MRN complex responded to every type of lesion and at every time point investigated, the response of RPA to MMC was very weak and only occurred at the latest time point investigated despite the robust MRN response. The difference in response of the MRN complex and RPA is also supported by the observation that, although both the MRN complex and RPA responded to ETOP, their responses were independent of each



**FIG. 1. HU-, CAMPT-, ETOP-, and MMC-induced foci formation.** HeLa cells were treated for 3 h with 4 mM HU, 1  $\mu$ M CAMPT, 25  $\mu$ M ETOP, and 1  $\mu$ g/ml MMC or mock-treated. Following fixation in 4% paraformaldehyde, the cytoplasmic and nucleoplasmic proteins were extracted with PBS containing 0.5% Triton X-100 and the cells were incubated in primary and secondary antibodies and visualized by confocal microscopy. *A*, panels *A–R*, HU- and CAMPT-induced phospho-Nbs1 and RPA foci co-localization (panels *F* and *I*). In contrast, ETOP-induced phospho-Nbs1 and RPA foci did not form in the same cells (panels *J–O*). Cells were either positive for phospho-Nbs1 foci or RPA foci but not both. MMC treatment induced the formation of phospho-Nbs1 foci but not RPA foci (panels *P–R*). *B*, quantification of the percentage of cells containing phospho-Nbs1, RPA, both phospho-Nbs1 and RPA foci, or neither following HU, CAMPT, ETOP, or MMC treatment. Cells treated with HU and CAMPT showed an increase from ~40% cells with phospho-Nbs1 and RPA foci to 55–60% as exposure time increased from 1 to 6 h. Treatment with ETOP led to the expression of either phospho-Nbs1 or RPA foci, but the percentage of cells with both types of foci remained much lower. Treatment with MMC led to increasing percentage of cells with phospho-Nbs1 foci with time and a small percentage of cells with both phospho-Nbs1 and RPA foci at 6 h.

other as demonstrated by the ability of each to form foci in the absence of the other.

**HU, CAMPT, ETOP, and MMC Induce RPA-p34 Hyperphosphorylation**—We have previously shown that HU treatment leads to damage-dependent phosphorylation of Mre11 and hyperphosphorylation of RPA-p34 (15). Formation of phospho-Nbs1 foci in the immunofluorescence experiments verified that HU, CAMPT, ETOP, and MMC all induced the phosphorylation of Nbs1. To determine whether these agents induced hyperphosphorylation of RPA-p34, we performed Western immunoblotting of whole-cell lysates and looked for the characteristic hyperphosphorylated band. HU, CAMPT, ETOP, and MMC all led to

the hyperphosphorylation of RPA-p34 with a time-dependent increase in the amount of the hyperphosphorylated form (Fig. 2, lanes 2–4, 6–8, 10–12, and 16; Form 5 represents the hyperphosphorylated band). Consistent with the foci data that showed that RPA did not respond to MMC treatment until 6 h, RPA hyperphosphorylation was only seen at the 6-h time point and was much less robust than with the other agents (Fig. 2, lane 16).

**ETOP and MMC Induce DNA DSBs**—The foci data suggested that different types of lesions generated specific responses that differed in the extent of RPA involvement. One difference, comparing HU and CAMPT with ETOP and MMC

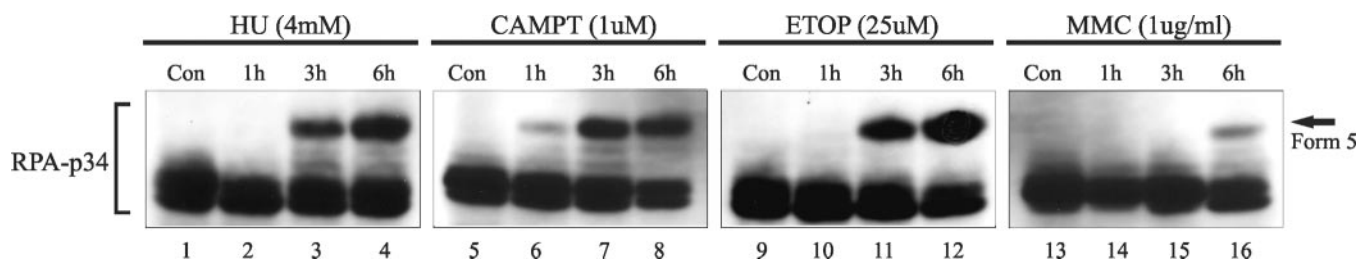


FIG. 2. HU-, CAMPT-, ETOP-, and MMC-induced hyperphosphorylation of RPA-p34. HeLa cells were treated with 4 mM HU, 1  $\mu$ M CAMPT, 25  $\mu$ M ETOP, or 1  $\mu$ g/ml MMC for 1–3 h. HU, CAMPT, ETOP, and MMC led to the hyperphosphorylation of RPA-p34 (lanes 2–4, 6–8, 10–12, and 16). RPA-p34 hyperphosphorylation was determined by the appearance of a slower migrating band (Form 5) on the Western blot.

treatment, is the generation of DNA DSBs. ETOP is known to be a potent inducer of DNA DSBs through the inhibition of topoisomerase II (54, 55), and repair of MMC-induced damage progresses through a DNA DSB intermediate (58–60). In contrast, HU leads to replication fork stalling due to depletion of deoxyribonucleotide substrates (44, 45) and camptothecin induces DNA SSBs through inhibition of topoisomerase I (47, 52). However, it must be noted that both HU and CAMPT have the potential to induce DNA DSBs, HU through the collapse of stalled replication forks (48–50) and CAMPT through the replication of nicked template DNA (47, 51, 52). To investigate the extent of DNA DSBs induced by these four agents, we utilized the neutral comet assay. The neutral comet assay allows for visualization and quantification of DNA DSBs from individual cells. The fold increase in the amount of DNA DSBs above non-damaged controls was compared for the different experimental groups using tail moment measurements obtained by computer analysis of comet images using CometScore™. A two-tailed Student's *t* test was used to compare the experimental to non-damaged control samples, and a *p* value of  $<0.02$  was considered significant. As expected, there was no difference in the amount of DNA DSBs in control cells and HU-treated cells (Fig. 3A). Even though CAMPT treatment led to an apparent 3-fold increase in the amount of DNA DSBs (Fig. 3B), only the 1-h time point was significantly different from the non-damaged controls (1 h, *p* = 0.014; 3 h, *p* = 0.126; and 6 h, *p* = 0.041). There was a time-dependent increase in ETOP-induced DSBs culminating in a 7-fold increase over controls (1 h, *p* = 0.002; 3 h, *p* = 0.001; and 6 h, *p* = 0.005). Likewise, MMC-induced DSBs culminated in a 6-fold increase in DSBs over controls by 6 h (1 h, *p* = 0.172; 3 h, *p* = 0.051; 6 h, *p* = 0.014) (Fig. 3, C and D). These data suggested that the presence of DNA DSBs may play a role in the loss of co-localization of the MRN complex and RPA.

*Prolonged HU Exposure Induces DNA DSBs and Loss of Co-localization of Phospho-Nbs1 and RPA Foci*—Short-term HU exposure causes replication fork arrest and co-localization of phospho-Nbs1 and RPA foci. Prolonged HU exposure induces DNA DSBs through the collapse of stalled forks. Therefore, we utilized such prolonged HU exposure to determine whether the co-localization results were dependent on the presence or extent of DSBs (48–50). After 24 h of continuous HU treatment, cells contained similar amounts of DSBs as ETOP- and MMC-treated cells (Fig. 4A). At the immunofluorescence level, all of the HU-treated cells contained both phospho-Nbs1 and RPA foci; however, the foci no longer co-localized as seen at earlier time points (Fig. 4B). The loss of co-localization in the prolonged HU-treated cells together with DNA DSBs induced by ETOP and MMC, leading to a very small percentage of foci-positive cells expressing both phospho-Nbs1 and RPA foci, suggests that the MRN complex and RPA respond to DNA DSBs independently of one another.

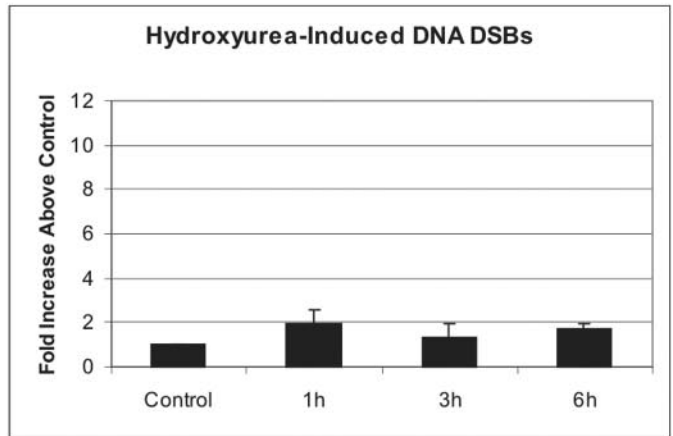
## DISCUSSION

Stalled replication forks induced by HU treatment and DNA SSBs induced by CAMPT both activate the DNA damage response in a similar manner with the phosphorylation of Nbs1, hyperphosphorylation of RPA-p34, and the formation of foci containing both the MRN complex and RPA. DNA DSBs induced by ETOP or MMC treatment also activated the DNA damage response; however, the involvement of the MRN complex and RPA was distinctly different from that seen with HU and CAMPT. In contrast to the HU- and CAMPT-induced response, ETOP induced the phosphorylation of Nbs1 and hyperphosphorylation of RPA and foci that contained either phospho-Nbs1 or RPA but not both. In further contrast, MMC treatment induced the phosphorylation of Nbs1 and formation of phospho-Nbs1 foci but not hyperphosphorylation and foci formation of RPA. This finding suggested that the activation and localization of the MRN complex and RPA were dependent on the type of lesion. Lesions that contained stalled replication forks or SSBs induced activation and co-localization of the MRN complex and RPA, but lesions that contained cross-linked DNA or DSBs induced a different RPA response.

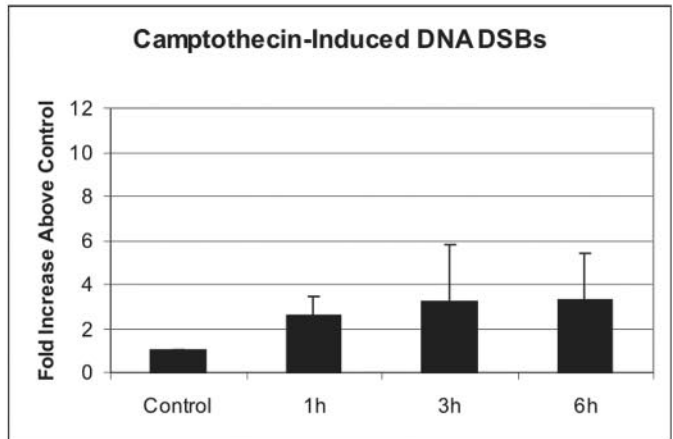
The response to DNA DSB leads to the activation of one of two different pathways to repair the damaged DNA: 1) non-homologous end joining or 2) homologous recombination. The Rad52 epistasis group of proteins plays important and critical roles in homologous recombination. It has been shown previously that both Rad51 (member of the Rad52 epistasis group) and Mre11 form foci following ionizing radiation treatment but that Rad51 and Mre11 foci were not found in the same cell (16). Maser *et al.* (16) suggest that these data support the idea that the MRN complex and Rad51 have independent roles in DSB repair (16). More recently, Lisby *et al.* (3) showed that the protein composition of foci changed dynamically at the site of a single endonuclease-induced DSB. Mre11/Rad50/Xrs1, the yeast MRN complex, formed foci very early at the site of the break, but Mre11/Rad50/Xrs1 had disappeared from the foci by the time Rad51 and Rad52 were recruited to the same site (3). In that study, they also demonstrated that RPA was recruited to the site of the break before the departure of Mre11/Rad50/Xrs1 and that the formation of Rad51 and Rad52 foci at the site of damage was dependent on the presence of RPA. With these facts in mind, we investigated the role of DNA DSBs in the observed differences in phospho-Nbs1 and RPA foci co-localization.

Treatment with either ETOP or MMC led to DNA DSBs and to phospho-Nbs1 foci in the absence of RPA foci. To determine whether previously co-localized phospho-Nbs1 and RPA foci at stalled forks continued to co-localize following collapse of the stalled fork and generation of a DSB, prolonged HU treatment of cells was employed. After 24 h of continuous exposure to HU, cells contained amounts of DNA DSBs comparable with those induced by ETOP and MMC treatment. At this later time point, phospho-Nbs1 and RPA foci were both still present in the

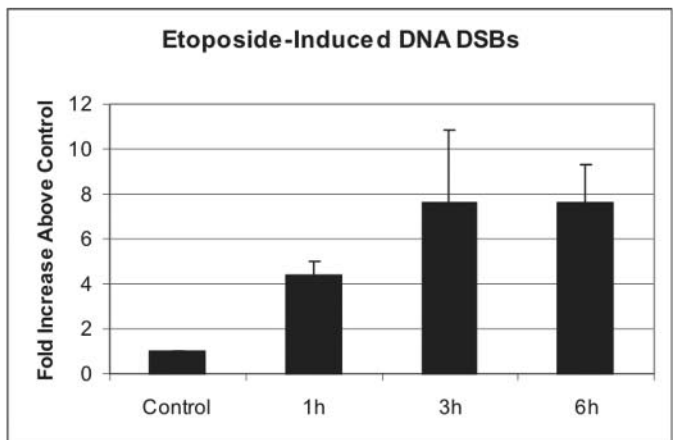
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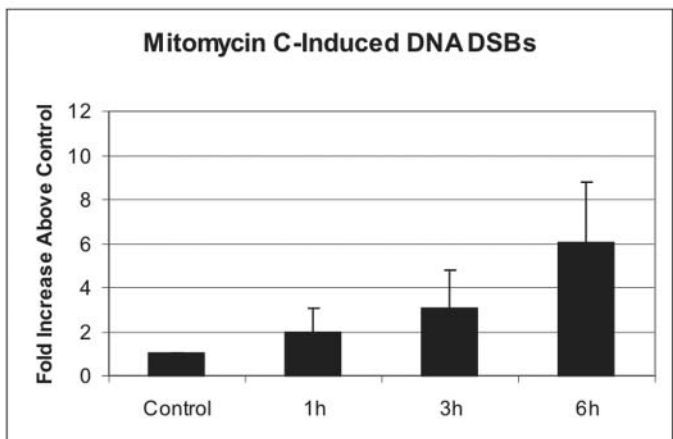
B.



C.



D.



**FIG. 3. ETOP- and MMC-induced generation of DNA DSBs.** HeLa cells treated with HU, CAMPT, ETOP, and MMC or mock-treated were analyzed for the presence of DNA DSBs using the neutral comet assay. Results were presented as fold increase of DNA DSBs above non-damaged controls. *A*, 4 mM HU treatment did not induce DNA DSBs above control levels at the time points presented. *B*, 1  $\mu$ M CAMPT induced an apparent 3-fold increase in DSBs but overall was not statistically different from non-damaged controls. *C*, 25  $\mu$ M ETOP treatment induced a time-dependent increase in DSBs with an  $\sim$ 7-fold increase in DNA DSBs at 6 h. *D*, 1  $\mu$ g/ml MMC treatment led to a time-dependent increase in the amount of DNA DSBs culminating at a 6-fold increase in DSBs.

A.

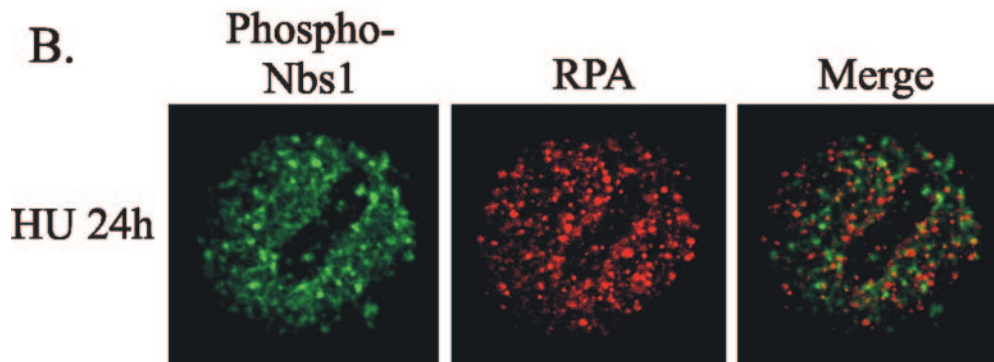
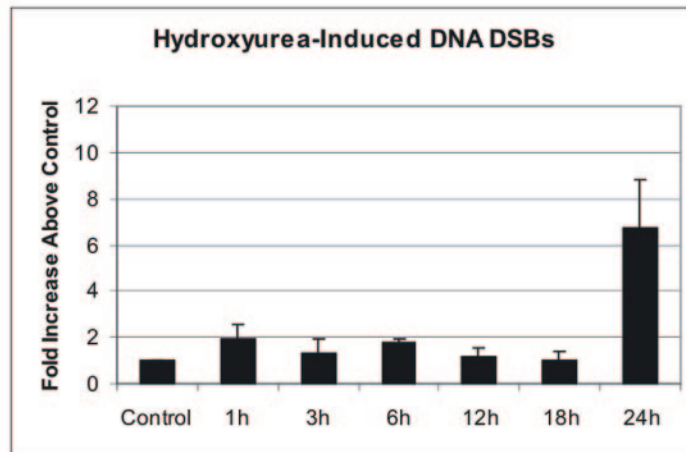


FIG. 4. Prolonged exposure to HU induced DNA DSBs and loss of phospho-Nbs1 and RPA foci co-localization. *A*, a time course of prolonged HU treatment showed that, at 24 h of continuous exposure to 4 mM HU, cells had DNA DSBs equivalent in amount to cells treated with ETOP or MMC. *B*, at 24 h of continuous 4 mM HU treatment, phospho-Nbs1 and RPA foci were visualized by confocal microscopy. Cells contained both phospho-Nbs1 and RPA foci, but these foci no longer co-localized.

nucleus but they no longer co-localized. This finding suggested that the transition from stalled replication fork to DNA DSB resulted in the loss of co-localization of the MRN complex and RPA. This loss of co-localization may represent independent functions of the MRN complex and RPA in the repair of DSBs or may represent a “snapshot” of the temporally dynamic composition of foci as reported by Lisby *et al.* (3).

The type of DNA damage may play a critical role in determining whether RPA is bound to the damaged DNA, which in turn affects the ability of RPA to become hyperphosphorylated (63, 64). The binding of RPA to ssDNA has been well characterized. A 30-nucleotide-long stretch of ssDNA is required for usual RPA binding (65, 66), but under certain circumstances, RPA can bind with only 8–10 nucleotides (63, 67). The 8-nucleotide binding mode is probably a precursor to the more stable 30-nucleotide binding (63) and is consistent with the observed lower affinity of RPA for shorter oligonucleotides *in vitro* (68). In our assays, the lack of RPA foci only indicates that the RPA content in the foci is below the level of resolution of this assay and cannot be taken to mean that RPA is completely absent. Therefore, the differences in RPA binding are probably due to relative amounts. With HU treatment, large sections of ssDNA are generated at the sites of stalled forks (44), providing

a substrate for RPA binding. With CAMPT treatment, the inhibition of topoisomerase I allowed for continued replication and the generation of additional ssDNA. Inhibition of topoisomerase II by ETOP leads to the production of DNA DSBs, which may not have significant amounts of ssDNA at the broken ends initially. The MRN complex is probably recruited to these sites to tether the broken ends together (69, 70) and then help recruit other necessary protein factors including a nuclease that processes the ends to generate ssDNA that is then bound by RPA (3). Interstrand cross-linking may prevent the accumulation of significant ssDNA, because the interstrand tethering would inhibit DNA unwinding and thus prevent the formation of a ssDNA platform to which RPA could bind. The MMC experiments presented here, which demonstrated a lack of RPA foci formation and RPA hyperphosphorylation, are consistent with such a model. This lack of a response by RPA also occurs with cisplatin, another agent that causes interstrand cross-links,<sup>2</sup> indicating that there is something about the interstrand cross-link that either inhibits RPA binding and activation or is not recognized by a signaling and repair pathway that includes RPA.

<sup>2</sup> J. Turchi, personal communication.

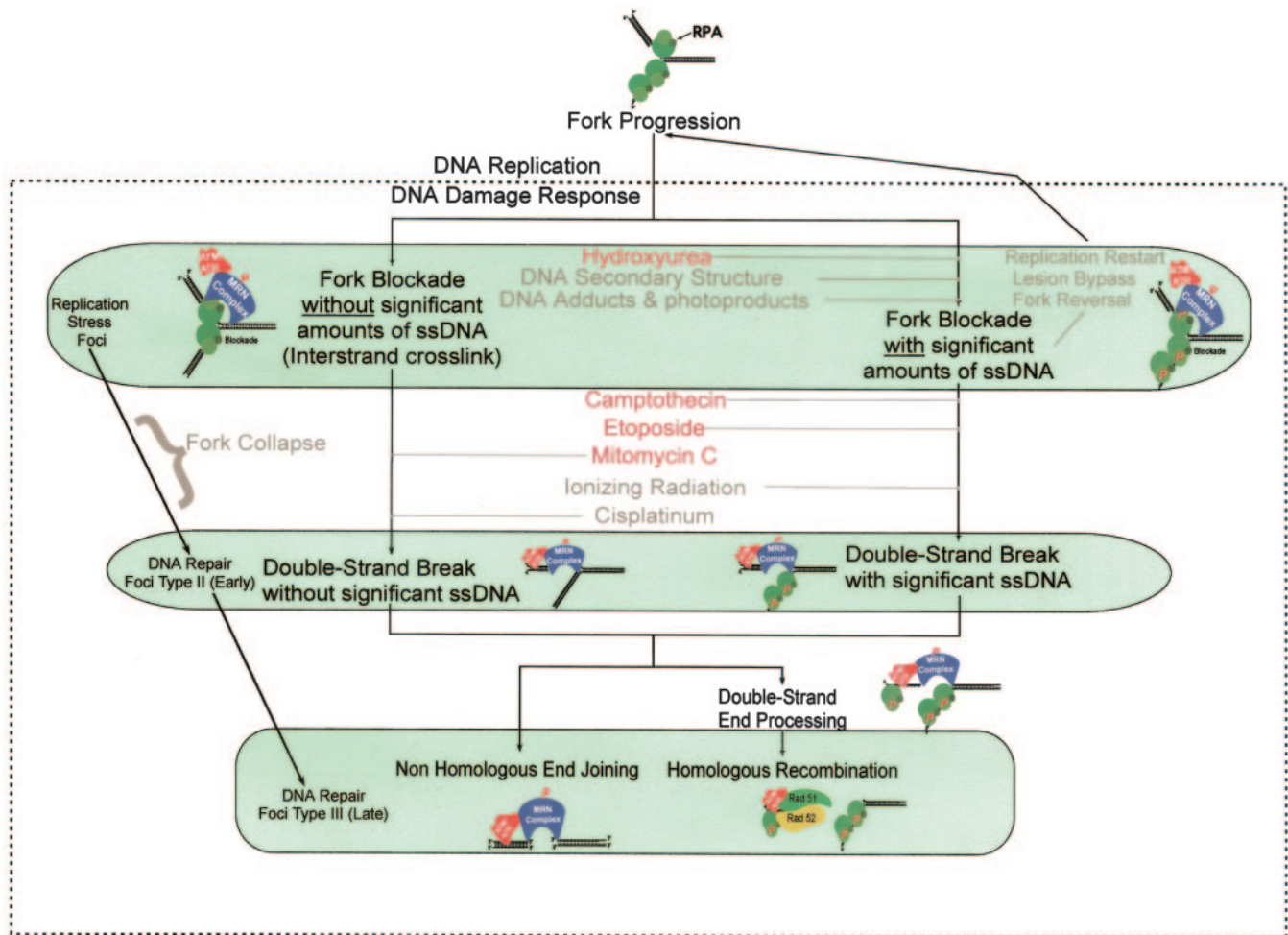


FIG. 5. Model of the interaction of the MRN complex and RPA in response to different DNA lesions (see “Discussion” for details).

These results, combined with observations made by others, are compatible with a model of DNA repair that considers the availability of ssDNA for RPA binding and subsequent hyperphosphorylation (Fig. 5) to explain the foci data presented here. This model depicts the MRN complex as a sensor for damage and bestows a protector, sensor, and effector function on RPA. RPA protects ssDNA and is hyperphosphorylated to identify replication stress, SSBs, and some types of DSBs. The replication stress foci, if unrepaired, lead to replication fork collapse and subsequent DNA DSBs and the formation of early DNA repair foci (similar to Type II foci described by Mirzoeva and Petrini (18)). These type II foci contain MRN and can also contain RPA depending on the type of DNA damage and amount of ssDNA. Subsequently, the foci evolve into type III DNA repair foci that represent repair of DSBs either through a recombinatorial pathway using Rad51/Rad52 or non-homologous end joining. These processes of DSB repair may occur within the same cell or different cells, thus leading to differences in protein composition of the foci.

We have demonstrated that the MRN complex responds to multiple types of lesions and that RPA is involved in response to a subset of these lesions, probably those that contain significant stretches of ssDNA. Additionally, these data demonstrate that the MRN complex and RPA can act independently of each other and probably have different roles in the repair of DSBs that are separated physically and/or temporally. However, these data only include a small proportion of time points and agents that could be investigated to further elucidate the nature of the MRN complex and RPA interaction and the impor-

tance of that interaction on repair of specific types of lesions. Ongoing work to quantitate the amount of ssDNA present in the different lesions will help determine whether this is the explanation for the presence or absence of RPA foci and the unexpected absence of RPA in the response to interstrand cross-linking agents.

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