Potential Role of MLH1 in the Induction of p53 and Apoptosis by Blocking Transcription on Damaged DNA Templates

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Abstract
Defects in DNA mismatch repair (MMR) are common in human cancers, confer tolerance to certain types of chemotherapeutic agents, and lead to genomic instability. In addition to their mismatch-correcting roles during DNA replication, MMR proteins can bind to certain DNA lesions and signal p53 and apoptosis by an unknown mechanism. To further study the mechanism by which the MMR protein MLH1 is involved in the induction of p53 and apoptosis, we exposed the colon carcinoma cell line HCT116 (MLH1-deficient) and mlh1-corrected HCT116 sublines to alkylating agents or hydrogen peroxide (H₂O₂). It was found that while alkylating agents induced both apoptosis and phosphorylation of the Ser-15 site of p53 in a MLH1-dependent manner, induction of apoptosis, but not p53 phosphorylation, was MLH1 dependent following treatment with H₂O₂. The MLH1-dependent induction of p53 phosphorylation by alkylating agents did not appear to be cell cycle dependent, arguing against a futile repair mechanism operating during S phase as the sole mechanism for the MLH1-dependent DNA damage signaling. Importantly, we found that both alkylating agents and H₂O₂ caused significant inhibition of mRNA synthesis in MLH1-expressing but not in MLH1-deficient cells. These findings suggest a novel mechanism of MLH1 in the induction of p53 and apoptosis by inhibiting RNA polymerase II-dependent transcription on damaged DNA templates.

Introduction
DNA mismatch repair (MMR) proteins play an important role in repairing mismatches caused by DNA polymerase errors (1). In addition to mismatch correction, it is thought that MMR proteins participate in the regulation of both mitotic and meiotic recombination (1, 2) and in transcription-coupled repair (3), although their roles in transcription-coupled repair has been disputed (4, 5). Furthermore, it has been shown that MMR proteins can bind to certain DNA lesions such as O²-methylguanine, 8-hydroxyguanine, and N-acetoxy-N-acetyl-2-aminofluorene and that this binding is linked to the induction of p53 and apoptosis (6–9). The loss of MMR in tumor cells is thought to give these cells a selective advantage (10) and subsequently would make the tumor more resistant to the induction of apoptosis by anticancer therapy. In fact, MMR-deficient cells have been shown to be tolerant to many types of anticancer agents such as cisplatin (11), alkylating agents (12), methotrexate (13), DNA topoisomerase II inhibitors (14), oxidative stress (15, 16) and 5-fluorouracil (17).

The role of MMR proteins in the induction of p53 and apoptosis following induction of DNA damage is not yet understood, but at least three different mechanisms have been proposed (18–20). First, replication past a damaged nucleotide may activate MMR proteins to remove the newly synthesized strand opposite the damaged nucleotide. This would lead to a futile cycle of nicking and rejoining, which could subsequently lead to the formation of double-strand breaks and the induction of p53 and apoptosis. Second, MMR proteins may be involved in preventing recombinational repair of double-strand breaks induced by replication blockage at sites of DNA lesions (19). A third mechanism, i.e., not necessarily replication dependent, suggests that MMR proteins may bind DNA lesions and form DNA damage signaling complexes capable of inducing p53 and apoptosis (20).

In this study, we investigated whether the MMR protein MLH1 may trigger the induction of p53 and apoptosis following treatment with alkylating or oxidative stress-inducing agents by blocking transcription. The rationale of our study was that we have previously shown that there is a strong correlation between the blockage of transcription and the induction of p53 and apoptosis (21–28). Using the MMR-deficient colorectal cancer cell line HCT116 and MMR-corrected sublines containing either an extra copy of the human chromosome 3 on which the mlh1 gene resides (12) or a vector containing the mlh1 gene (29), we show that transcription is inhibited in a MLH1-dependent manner in cells treated with the alkylating agents N-methyl-N-nitrosourea (MNU) and N-methyl-N²-nitro-N-nitrosoguanidine (MNG). Importantly, this inhibition correlated with the induction of p53 phosphorylation and apoptosis. Furthermore, induction of apoptosis following exposure to the oxygen radical-inducing agent hydrogen...
peroxide (H₂O₂) correlated to inhibition of mRNA synthesis. Based on these results, we propose that MLH1 triggers the induction of p53 and apoptosis by blocking transcription on damaged DNA templates.

**Results**

**MLH1-Dependent Induction of Apoptosis and Phosphorylation of p53 by Alkylating Agents**

The alkylating agents MNNG and MNU induce, among other lesions, O⁶-methylguanine in cellular DNA (30). This type of lesion is mutagenic, toxic, and normally repaired by the O⁶-methylguanine methyl transferase, an enzyme that can be inhibited by O⁶-benzylguanine (O⁶-BG) (31, 32). MMR proteins have been shown to bind to O⁶-methylguanine adducts (33), and this binding is thought to trigger the induction of p53 and apoptosis (6–9). However, the mechanism by which MMR proteins trigger p53 and apoptosis following exposure to alkylating agents is unknown.

To investigate the potential mechanism of the MLH1-dependent induction of p53 and apoptosis by alkylating agents, we used the colorectal cancer cell line HCT116 (MLH1-deficient) and MMR-corrected HCT116 cell clones transfected with either a copy of the human chromosome 3 (HCT116.ch3) (12) or the human mlh1 gene (29). To verify that alkylating agents induce apoptosis in a MLH1-dependent manner in this cell system, we treated the MLH1-deficient and MLH1-expressing HCT116 cells with either MNNG or MNU for 1 h in the presence of O⁶-BG and then scored apoptosis 72 h later. At the doses used, these treatments resulted in significant induction of apoptosis in MLH1-proficient but not in MLH1-deficient cells (Fig. 1A). These results are in accordance with previous reports (6–9). Furthermore, the induction of apoptosis was mediated by O⁶-methylguanine lesions because it was only observed in cells in which O⁶-methylguanine methyl transferase activity had been inhibited by O⁶-BG. The MLH1-proficient and MLH1-deficient cells were similarly sensitive to the induction of apoptosis by actinomycin D, suggesting that the MLH1-proficient cells were not hypersensitive to apoptotic activators in general.

It was recently shown that the induction of phosphorylation of the Ser-15 site of p53 following treatments with MNU or MNNG, but not UV light or etoposide, is dependent on MSH2 and MLH1 (7). In that study, the induction of Ser-15 phosphorylation was observed at 18 h and persisted for at least 72 h. In this study, we examined the early kinetics of the induction of Ser-15 phosphorylation in the MLH1-proficient and MLH1-deficient HCT116 cells and found that Ser-15 phosphorylation following MNU treatment was MLH1 dependent as previously reported (7). Furthermore, Ser-15 phosphorylation of p53 could be detected as early as 4 h after treatment (Fig. 1B). No induction of Ser-15 phosphorylation was observed in the MLH1-deficient cells.

**Induction of Ser-15 Phosphorylation by MNU Do Not Appear to be Cell Cycle Dependent**

It has been proposed that the signal that triggers the induction of p53 following exposure to alkylating agents originates from DNA strand breaks generated through futile attempts by MMR proteins to remove the newly synthesized strand opposite O⁶-methylguanine adducts. If this model is correct, then cells must traverse the S phase following the induction of DNA damage in order for p53 to get induced. To explore whether the p53 phosphorylation in MLH1-proficient cells following exposure to MNU is cell cycle dependent, we used dual-labeling flow cytometry to measure Ser-15 phosphorylation as a function of the cell cycle phase in unsynchronized cells. Although the Ser-15 phosphospecific anti-p53 antibodies gave a very distinct and strong band when used for Western blotting, this antibody gave a weaker signal when using flow cytometry. We observed a shift representing a 20–40% increase in the mean antibody FITC signal for the treated sample compared with untreated controls. Importantly, this shift was not confined to a certain phase of the cell cycle; rather, we observed that phosphorylation of p53 at Ser-15 increased in all phases of the cell cycle following MNU treatment (Fig. 2A). Although slightly more Ser-15

![Figure 1](https://example.com/figure1.png)

**FIGURE 1.** MLH1-dependent induction of apoptosis and phosphorylation of p53 by alkylating agents. A, HCT116 and HCT116 + vector (MLH1-deficient) and HCT116.ch3 and HCT116 + mth1 (MLH1-proficient) cells were exposed to 10-μM MNNG, 2-mM MNU, or 200-nM actinomycin D (act D), and the amount of apoptosis was assessed by flow cytometry 72 h later. Legend: light gray bars, MLH1-deficient cells; black bars, MLH1-proficient cells; dark gray bars, MLH1-proficient cells treated with MNU in the absence of O⁶-BG. Columns, average of three to five experiments; bars, SEM. B, HCT116 and HCT116 + vector (MLH1-deficient) and HCT116.ch3 and HCT116 + mth1 (MLH1-proficient) cells were exposed for 1 h to 2-mM MNU (in the presence of O⁶-BG), and the phosphorylation of p53 at the Ser-15 site was assessed at 0, 4, or 8 h following treatment. Phosphorylation of the Ser-15 site of p53 was assessed by immunoblotting with a phosphospecific anti-p53 antibody. The Coomassie blue staining pattern for total proteins on the membranes showed equal loading.
phosphorylation was found in the S and G2/M phases of the cell cycle, the fact that cells in the G1 phase also triggered Ser-15 phosphorylation suggests that this function of MLH1 is not exclusively S phase dependent. Although it cannot be excluded that the cells found in the G1 phase of the cell cycle at the time of fixation had traversed the S and G2/M phases during the 8-h postincubation period, this possibility is unlikely because alkylating agents induce a pronounced G2 arrest in these cells (34). However, to further investigate whether the MLH1-dependent induction of Ser-15 phosphorylation of p53 is S phase dependent, we performed flow cytometry experiments with cells that had been incubated in the presence of bromodeoxyuridine (BrdUrd) during and after the treatment with MNU. BrdUrd specifically labels cells that are traversing the S phase, and by using anti-BrdUrd antibodies, the cell samples could be divided into a population of cells that traversed S phase (BrdUrd+) and a population that did not traverse S phase (BrdUrd−) during the 16-h postincubation period. Using this approach and flow cytometry, it was found that following treatment with MNU, the shift in Ser-15 phosphorylation of p53 occurred in both BrdUrd− and BrdUrd+ cells (Fig. 2B). The shift in Ser-15 phosphorylation following treatment was found to be significant in both BrdUrd− and BrdUrd+ populations. Taken together, these findings suggest that the involvement of MLH1 in signaling p53 phosphorylation is not restricted to the S phase of the cell cycle and argues against an exclusive role of a MMR-directed futile repair mechanism during the S phase in the induction of Ser-15 phosphorylation of p53.

**MLH1-Dependent Suppression of Messenger RNA Synthesis Following Treatment With Alkylating Agents**

To further explore possible mechanisms for the MLH1-dependent induction of p53 and apoptosis, we tested whether alkylating agents may inhibit general mRNA synthesis in a MLH1-dependent manner. It has previously been shown that the induction of p53 and apoptosis following UV irradiation is linked to blockage of transcription (21–24, 28, 35–38). It is possible that RNA polymerase II complexes may serve as sensors of DNA damage that when blocked at DNA lesions signal p53 and apoptosis as well as repair proteins involved in transcription-coupled repair (25). While UV light-induced cyclobutane pyrimidine dimers and 6-4 photoproducts efficiently block the elongation of RNA polymerase II in vitro (39, 40), the premutagenic DNA lesion O6-methylguanine have been shown to be efficiently bypassed by RNA polymerases in vitro (41).

To test the hypothesis that the MLH1 protein may play a role in DNA damage signaling by interacting with DNA lesions and blocking transcription, we investigated what effect alkylating agents had on the synthesis of mRNA in the MLH1-proficient and MLH1-deficient HCT116 cells. Our results show that mRNA synthesis was not significantly affected in the MMR-deficient HCT116 cells following treatment with MNNG or MNU in the presence of O6-BG (Fig. 3). This suggests that O6-methylguanine lesions do not inhibit transcription directly in the absence of MLH1. In the MLH1-corrected cells, mRNA synthesis was significantly inhibited 4 h following treatment. We used the transcription inhibitor actinomycin D as a control and found equal inhibition of transcription in both cell lines (data not shown). In the absence of O6-BG, no inhibition of transcription was observed in the MLH1-proficient cells treated with MNU, strongly suggesting that O6-methylguanine lesions were responsible for the reduced mRNA synthesis in the MLH1-corrected cells. Time course experiments revealed that inhibition of transcription was transient and maximal at 4 h after treatment (data not shown). This suggests that transcription blocking may result from a time-dependent MMR complex formation at the sites of O6-methylguanine lesions in the transcribed strand of active genes and that the blockage is eventually resolved. Taken together, these results show that O6-methylguanine lesions do not by themselves inhibit transcription, but in the presence of a functional MMR system, mRNA synthesis is significantly reduced.

**FIGURE 2.** MLH1-dependent phosphorylation of p53 is not cell cycle dependent. A. HCT116 ch3 cells were treated with 2 mM MNU as described in Fig. 1, and cells were collected and fixed 8 h later. The amount of Ser-15 phosphorylation as a function of the cell cycle was determined by dual-labeling flow cytometry of the fixed cells stained with phosphospecific anti-p53 antibodies and propidium iodide. Columns, increased amount of Ser-15 antibody reactivity in MNU-treated cells above untreated controls and average of three different experiments; bars, SEM. B. HCT116 ch3 cells were treated with 2 mM MNU and incubated with 33.3 μM BrdUrd during and for 16 h following MNU treatment to specifically label cells that progressed through S phase during the experiment. The data were gated as BrdUrd− and BrdUrd+ cells and plotted as number of cells against level of FITC signal (Ser-15-P signal). White plots, untreated cells; gray plots, MNU-treated cells. This experiment was performed nine times, and the mean of the average Ser-15 phosphorylation signal increased 38% and 37% for BrdUrd− and BrdUrd+, respectively. These increases were statistically significant with P < 0.005 (BrdUrd−) and P < 0.001 (BrdUrd+).
Induction of Apoptosis, but not p53, Correlates to MLH1-Dependent Inhibition of Messenger RNA Synthesis Following Treatment With H$_2$O$_2$

It has been previously shown that, in addition to alkylating agents, MMR-deficient cells are more tolerant than corresponding MMR-proficient cells to the oxidative stress-generating agents ionizing radiation (low-dose rate) (16), H$_2$O$_2$, and tert-butyl hydroperoxide. (15, 42). To confirm these studies using our cell system, we treated cells with the hydroxyl radical-inducing agent H$_2$O$_2$ and found that apoptosis was induced to a much greater extent in the MLH1-proficient cells than in the MLH1-deficient cells (Fig. 4A). We next investigated the role of MLH1 in the induction of Ser-15 phosphorylation following treatment with H$_2$O$_2$ and found that Ser-15 phosphorylation occurred in both MLH1-proficient and MLH1-deficient cells (Fig. 4B). Furthermore, the phosphorylation was rapid and evident directly after the 1-h H$_2$O$_2$ treatment. This finding may be unexpected because the MLH1-proficient cells were clearly more prone to undergo apoptosis following H$_2$O$_2$ treatment than were the MLH1-deficient cells (Fig. 4A). However, the MLH1-independent induction of Ser-15 phosphorylation could be explained by the significant number of DNA strand breaks induced by H$_2$O$_2$, resulting in the phosphorylation of the Ser-15 site via a MLH1-independent pathway involving the ATM kinase (43–45).

Thymine glycols and 8-hydroxyguanine are two biologically important types of lesions induced by ionizing radiation or treatment with H$_2$O$_2$ (46). Studies using transcription assays with DNA templates containing site-specific lesions suggest that RNA polymerases can bypass both of these types of lesions in vitro (41, 47, 48). However, 8-hydroxyguanine adducts have been shown to inhibit transcription from plasmids in human cells (49). Thus, there may be specific factors in cells that bind these lesions and block transcription (47). To directly examine whether oxidative stress may inhibit endogenous mRNA synthesis from genomic DNA and whether a functional MMR status is required for such inhibition, we treated the MMR-deficient and proficient HCT116 cells for 1 h with 400-$\mu$M H$_2$O$_2$ and measured mRNA synthesis 4 h later. It was found that H$_2$O$_2$ treatment had no significant effect on mRNA synthesis in the MMR-deficient HCT116 cells, suggesting that H$_2$O$_2$-induced DNA lesions, such as thymine glycols and 8-hydroxyguanines, do not by themselves block transcription (Fig. 4C). In contrast, the same treatment of the MMR-corrected HCT116...
cells resulted in significant inhibition of mRNA synthesis (40–50%). These results suggest that, similarly to $O^6$-methylguanine lesions, $H_2O_2$-induced DNA lesions result in significant inhibition of transcription in a MLH1-dependent manner. Thus, these findings may explain the earlier findings showing that transcription of plasmids containing site-specific 8-hydroxyguanine lesions is blocked in cells (49) but not *in vitro* (41, 47, 48).

**Discussion**

In this study, we explored the mechanism by which MMR proteins may trigger DNA damage signaling following exposure to the alkylating agents MNNG and MNU and the oxygen radical-inducing agent $H_2O_2$. We show that both the induction of apoptosis and the phosphorylation of the Ser-15 site of p53 following exposure to alkylating agents were dependent on functional MLH1 (Fig. 1) as previously reported (6–9). Importantly, we observed that the MLH1-dependent phosphorylation of the Ser-15 site of p53 following treatment with MNU occurred within 4 h and in all phases of the cell cycle (Fig. 2). These results lend support to a model in which the MLH1-mediated induction of p53 by MLH1 is not dependent on replication. Thus, the “futile repair” model suggesting that induction of p53 and apoptosis is triggered by the attempted mismatch correction of the newly synthesized strand opposite damaged bases cannot fully explain this cell cycle-independent induction of p53.

We have previously shown that there is a connection between blocked transcription and induction of p53 and apoptosis (21–28). To test the hypothesis that $O^6$-methylguanine and hydroxyl radical-induced DNA lesions may trigger the MLH1-dependent p53 and apoptosis by inhibiting transcription, we measured mRNA synthesis in MLH1-proficient and MLH1-deficient HCT116 cells. The results show that the MLH1-mediated induction of p53 and apoptosis following treatment with MNNG or MNU correlated to MLH1-dependent inhibition of transcription (Fig. 3). This MLH1-dependent inhibition was mediated by $O^6$-methylguanine adducts because no inhibition of mRNA synthesis was detected in MNU-treated cells in the absence of the $O^6$-methylguanine transferase inhibitor $O^6$-BG. Furthermore, treatment with $H_2O_2$ resulted in significant inhibition of mRNA synthesis in the MLH1-deficient but not in the MLH1-deficient HCT116 cells (Fig. 4C). However, phosphorylation of the Ser-15 site of p53 following $H_2O_2$ treatment was induced regardless of MLH1 status most likely due to the presence of DNA strand breaks inducing phosphorylation of p53 via the ATM kinase. Thus, the MLH1-dependent induction of apoptosis following treatment with $H_2O_2$ correlated with the inhibition of mRNA synthesis but not with the induction of p53. This finding is similar to what we have observed in human fibroblasts exposed to UV light or cisplatin where apoptosis closely correlated to failure to recover mRNA synthesis rather than to the induction of p53 (24, 26, 27).

It has been reported that ATM activation/activity following exposure to ionizing radiation is not dependent on the MMR status of cells (50). Our results using $H_2O_2$ would be consistent with this finding because Ser-15 phosphorylation occurred in both MLH1-proficient and MLH1-deficient cells. However, in agreement with a previous study (7), we found a clear dependence of MLH1 for the induction of Ser-15 phosphorylation of p53 following treatment of cells with MNU or MNNG in the presence of $O^2$-BG. A recent study found an ATM-dependent induction of Ser-15 phosphorylation of p53 following MNNG treatment that correlated to induction of DNA strand breaks (51). However, that study used higher doses of MNNG and did not include $O^6$-BG during treatment or the postincubation period. Thus, their results probably reflect base excision repair-induced DNA strand breaks leading to the activation of the ATM kinase.

Our results that no inhibition of transcription was observed in MLH1-deficient HCT116 cells following treatments with MNNG, MNU, or $H_2O_2$ suggest that $O^6$-methylguanine adducts or $H_2O_2$-induced lesions do not by themselves block transcription, which is in accordance with results obtained using *in vitro* transcription assays (41). However, mRNA synthesis was significantly reduced in the MLH1-proficient cells following exposures to either alkylating agents or $H_2O_2$. It has been shown that both $O^6$-methylguanine adducts (52, 53) and oxidative DNA adducts (49, 54) are subject to transcription-coupled base excision repair (tcBER). Furthermore, the MMR protein MSH2 has been suggested to be required in the tcBER of oxidative lesions (55). It is possible that the transcription-blocking activity of MLH1 at sites of $O^6$-methylguanine and oxidative DNA adducts may in addition to signaling p53 and apoptosis be responsible for the recruitment of base excision repair proteins for tcBER.

What is the mechanism by which MLH1 inhibits mRNA synthesis in cells exposed to alkylating agents or oxidative stress? One mechanism for the MLH1-dependent reduction in mRNA synthesis could be that MLH1 and other MMR proteins bind to DNA lesions and signal the down-regulation of general transcription initiation. However, this is unlikely because we found that p53 was phosphorylated at the Ser-15 site following MNU treatment, which we have previously shown is associated with inhibition of transcription elongation (28, 56). A more likely mechanism involves the binding of MLH1 and other MMR proteins to the lesions on DNA, which converts them into transcription-blocking lesions (Fig. 5). Alternatively, MLH1 proteins may travel with the RNA polymerase, and when encountering lesions in the transcribed strand, the MLH1 proteins may halt transcription leading to the phosphorylation of the Ser-15 site of p53.

Mismatch repair plays an important role in suppressing the formation of human tumors (57, 58). It is well established that the mismatch-correcting function of MMR proteins in the wake of DNA replication suppresses mutations and ensures genetic stability (18). However, the novel function of MLH1 as a transcription-blocking factor on damaged DNA templates described in this study may contribute to tumor suppression as well by causing the induction of p53, apoptosis, and perhaps tcBER. These results extend our model of RNA polymerase II as a sensor for DNA damage to also include lesions that do not directly block transcription but through the interaction with additional factors, such as MMR proteins, cause transcription blockage and subsequent induction of DNA damage signaling. This hypothesis provides a framework for
future studies searching for additional cellular proteins that may signal p53 and apoptosis by converting potentially mutagenic DNA adducts into apoptosis-inducing events through the blockage of transcription. Furthermore, these studies may lead to the development of new cancer therapeutic agents that either target transcription elongation directly or indirectly through the interaction with chemotherapy-induced DNA adducts.

Materials and Methods

Cell Lines

The human colorectal adenocarcinoma cell line HCT116 (MLH1 deficient) and a HCT116 hybrid clone containing an extra copy of chromosome 3 on which mlh1 resides (HCT116.ch3) were kindly provided to us by Dr. R. Boland (University of California at San Diego) (12). The HCT116 cell lines harboring an empty vector (HCT116 + vector) or a gene expressing the human mlh1 (HCT116 + mlh1) were kindly provided by Dr. F. Praz (Institut Gustave Roussy, Villejuif, France) (29). The cell lines were grown as monolayers in RPMI 1640 medium supplemented with 10% fetal bovine serum. To the media in which the HCT116.ch3 cells were growing, 400-μg/ml geneticin disulfate was added to select for cells carrying this extra chromosome. The expression of MLH1 in HCT116.ch3 and HCT116 + mlh1, or absence thereof in HCT116 cells, was verified by Western blot (data not shown).

Drug Treatments

Cells were preincubated in media containing 25-μM O6-BG for 2 h at 37°C to inhibit O6-methylguanine transferase activity. The cells were then exposed for 1 h at 37°C to 10-μM MNNG or 2-mM MNU in serum-free medium containing 25-μM O6-BG. Cells were washed and supplied with fresh medium containing 25-μM O6-BG and incubated at 37°C for different periods of time. For treatments with H2O2, the cells were exposed for 1 h at 37°C to 400-μM H2O2 in serum-free medium. The cells were then washed and supplied with fresh media and incubated at 37°C.

Measurement of Messenger RNA Synthesis

Cells were seeded in growth media containing 185-Bq/ml [14C]thymidine to uniformly label cellular DNA for 24 h. Cells were then treated with MNNG, MNU, or H2O2 for 1 h as described above. Following a 4-h incubation in fresh media containing O6-BG, nascent RNA was labeled for 1 h by adding [3H]uridine (7.5 × 106 Bq/ml) and measured as previously described (22, 56). Briefly, the labeled cells were rinsed twice in ice-cold PBS, detached by scraping, collected by centrifugation, and lysed using a lysis buffer from the straight A’s mRNA isolation system (Novagen, Madison, WI). Polyadenylated RNA was isolated using polydeoxythymidylic acid-conjugated magnetic beads (Novagen), and total nascent RNA synthesis was measured by precipitating cell lysates with an equal volume of 10% ice-cold TCA. The [3H] and [14C] counts present on the beads (mRNA) or on the filters (total RNA) were counted in a scintillation counter using a dual-counting program. Relative total RNA and polyadenylated acid RNA synthesis was then determined by calculating the ratio of [3H]/[14C] for each sample and comparing it with the ratio from an untreated control sample. The data are presented as the percentage of the [3H]/[14C] ratio for each treatment compared with the value determined from untreated control cells.

Western Blot

Cell lysates (30 μg) were subjected to SDS-PAGE and Western blot as previously described (28). The membranes were immunoblotted with Ser-15 phosphospecific anti-p53 antibody (Cell Signaling Technology, Beverly, MA). Equal loading and transfer of total proteins was confirmed by Coomassie blue staining of the membranes following Western blot.

Measurement of Apoptosis

Attached and floating cells were collected and analyzed for sub-G1 DNA content using flow cytometry as previously described (59).

Measurement of Ser-15 Phosphorylation of p53 in the Cell Cycle

Cells were pretreated for 2 h with 25-μM O6-BG followed by treatment with 2-mM MNU for 1 h at 37°C in serum-free medium containing 25-μM O6-BG. The cells were then washed and supplied with fresh medium containing 25-μM O6-BG and incubated at 37°C. In experiments involving BrdUrd, 33.3-μM BrdUrd was added concomitantly with the 2-mM MNU and was included throughout the experiments. Cells were then collected and fixed in 70% ethanol overnight. The fixed cells were pelleted and rinsed with HBT (5% fetal bovine serum and 0.5% Tween 20 in PBS) followed by incubation with Ser-15 phosphospecific anti-p53 antibodies with or without Alexa...
Fluor-conjugated anti-BrdUrd antibody (Molecular Probes, Eugene, OR) for 30 min. Cells were washed in HBT and resuspended and incubated with FITC-conjugated secondary antibodies. The cells were stained in a 0.5 ml ice-cold HBT solution containing propidium iodide (180 µg/ml) and RNase A (400 µg/ml) for 30 min at 4°C. The amount of propidium iodide staining and Alexa Fluor (BrdUrd) and FITC (Ser-15 phosphorylation) signal were analyzed on a single-cell basis using fluorescence-activated cell sorting Calibur two-parameter flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and Cell Quest software.

Acknowledgments
We thank Dr. Richard Boland for the generous gift of the HCT116 and HCT116.ch3 cells lines and Dr. Françoise Praz for the gift of the HCT116 +/+ vector and HCT116 +/+ cells. We thank the Flow Cytometry Core at the University of Michigan Comprehensive Cancer Center for technical assistance, Steve Kronenberg for help with some graphics, and Dr. Philip Hanawalt and the members of the Ljungman Laboratory for valuable discussions.

References


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1 NIH grant CA82376, a grant from the Gastrointestinal Oncology Program Fund of the University of Michigan Comprehensive Cancer Center, and a grant from the Biomedical Research Council at the University of Michigan Medical School.

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