

## Transcriptional Blockade Induces p53-dependent Apoptosis Associated with Translocation of p53 to Mitochondria\*<sup>§</sup>

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**The tumor suppressor p53 functions as a transcriptional activator to induce cell cycle arrest and apoptosis in response to DNA damage. Although p53 was also shown to mediate apoptosis in a manner independent of its transactivation activity, the mechanism and conditions that trigger such cell death have remained largely unknown. We have now shown that inhibition of RNA polymerase II-mediated transcription by  $\alpha$ -amanitin or RNA interference induced p53-dependent apoptosis. Inhibition of pol II-mediated transcription resulted in down-regulation of p21<sup>Cip1</sup>, which was caused by both transcriptional suppression and protein degradation, despite eliciting p53 accumulation, allowing the cells to progress into S phase and then to undergo apoptosis. This cell death did not require the transcription of p53 target genes and was preceded by translocation of the accumulated p53 to mitochondria. Our data thus suggested that blockade of pol II-mediated transcription induced p53 accumulation in mitochondria and was the critical factor for eliciting p53-dependent but transcription-independent apoptosis.**

Apoptosis is a genetically controlled mechanism of cell death that is essential for the elimination of unwanted cells during normal development and for the maintenance of tissue homeostasis. The tumor suppressor protein p53 induces either cell cycle arrest or apoptosis in response to a variety of cellular stresses, including DNA damage, oncogene activation, and hypoxia (1–3). Loss of p53 function therefore results in failure to remove damaged cells and contributes both to tumor development and to the resistance of cancer cells to therapies based on the induction of DNA damage.

It is well established that p53 is a transcriptional regulator and that p53-mediated apoptosis in response to DNA damage is predominantly attributable both to the transcriptional activation of genes that encode apoptotic effectors, such as the BH3-only proteins Noxa and PUMA, Bax, p53AIP1, and PERP

(4–9), as well as to the transcriptional repression of genes for antiapoptotic proteins such as Bcl-2 (10) and survivin (11). In addition to such transcriptional regulation, however, recent evidence has suggested the existence of a transcription-independent pathway of p53-mediated apoptosis (12, 13). A fraction of the p53 molecules that accumulate in damaged cells translocate to mitochondria, and this translocation is sufficient for p53 to induce permeabilization of the outer mitochondrial membrane through formation of complexes with the protective proteins Bcl-x<sub>L</sub> and Bcl-2, resulting in the release of cytochrome *c* into the cytosol (14, 15). The mechanism by which the relative activities of the transcription-dependent and transcription-independent pathways of p53-mediated apoptosis are regulated, however, has remained unclear.

Lesions in the transcribed strand of DNA induced by high concentrations of chemotherapeutic agents such as cisplatin or by high doses of UV radiation trigger the ubiquitination and consequent degradation of the large subunit (LS)<sup>1</sup> of RNA polymerase II (pol II), resulting in transcriptional collapse and the recruitment of DNA repair proteins (16, 17). A prolonged blockage of pol II-dependent transcription caused by failure to repair such DNA lesions results in cell death by apoptosis (18). Furthermore,  $\alpha$ -amanitin, a specific inhibitor of pol II-dependent transcription, also induces apoptosis (19). Given that pol II is responsible for the transcription of most protein-coding genes, new transcription of proapoptotic genes would not be expected to contribute to apoptosis induced by pol II inhibition or degradation. Although it is possible that such cell death is triggered by impaired expression of downstream survival factors, the precise mechanism of apoptosis elicited by transcriptional blockade remains unknown.

We have now investigated the molecular mechanism of apoptosis that occurs in response to transcriptional block caused by exposure of cells to  $\alpha$ -amanitin, RNA interference, or to a high dose of UV radiation. Inhibition of transcription resulted in accumulation of p53 and massive cell death both in normal fibroblasts and in HCT116 human colon carcinoma cells (*p53*<sup>+/+</sup>) without the induction of apparent DNA damage. Although new transcription of various p53 target genes was blocked as a result of pol II inhibition, the accumulation of p53

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<sup>1</sup> The abbreviations used are: LS, large subunit; CDK, cyclin-dependent kinase; DSB, DNA double-strand break; PCNA, proliferating-cell nuclear antigen; PI, propidium iodide; pol II, RNA polymerase II; pRb, retinoblastoma protein; BrdUrd, bromodeoxyuridine; siRNA, small interfering RNAs; RNAi, RNA interference; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; mAb, monoclonal antibody; RT, reverse transcription; ATM, ataxia telangiectasia-mutated; ATR, ATM and Rad3-related.

was required for apoptosis. We also found that a fraction of the accumulated p53 molecules, including those phosphorylated on Ser<sup>15</sup> or Ser<sup>46</sup>, translocated to mitochondria. In addition, we found that pol II inhibition induces the down-regulation of cyclin-dependent kinase inhibitor p21<sup>Cip1</sup> (p21<sup>WAF1</sup>), which is a target gene product of p53, allowing cells to enter S phase and to undergo apoptosis. Our observations support the notion that blockage of pol II-dependent transcription triggers p53-mediated, transcription-independent apoptosis.

#### MATERIALS AND METHODS

**Cell Lines and Cell Culture**—Parental (*p53*<sup>+/+</sup> and *p21*<sup>+/+</sup>), *p53*<sup>-/-</sup>, and *p21*<sup>-/-</sup> HCT116 cells (kindly provided by B. Vogelstein) and MRC-5 cells were cultured at 37 °C in Dulbecco's modified Eagle's medium/F-12 supplemented with 10% fetal bovine serum (without antibiotics) under a humidified atmosphere containing 5% CO<sub>2</sub>. Unless designated otherwise, HCT116 cells used in experiments were the parental cells. Cells were incubated with  $\alpha$ -amanitin (Sigma), MG132 (Calbiochem), adriamycin (Sigma), aphidicolin (Wako, Osaka, Japan), Z-Val-Ala-Asp(OMe)-fmk (Enzyme Systems Products, Livermore, CA), or actinomycin D (Sigma) as indicated.

**Expression Plasmids and siRNA Transfection**—A plasmid for expressing wild type p53 or a truncated form of p53 (p53( $\Delta$ 1–39), deletion of N terminus 39 amino acid) was constructed by subcloning the corresponding cDNA fragment into pcDNA3. These expression plasmids were transfected into cells using FuGENE 6 reagent (Roche Applied Science). Colonies were selected in complete medium containing G418 (800  $\mu$ g/ml; Invitrogen).

RNAi experiments were conducted as described previously (20). We used the following target sequence for pol II large subunit siRNA, 475'aactgaagcgaatgtctgtga<sup>495</sup>. The 21-nucleotide chimeric RNA-DNA duplexes were obtained from Japan BioServices Co., Ltd. (Saitama, Japan). Cells were transfected with annealed siRNAs with the use of Oligofectamine (Invitrogen). As a control we used the GL-2 duplex to target the luciferase gene (21).

**UV Irradiation**—Cells were washed with PBS, exposed to UV radiation (254 nm) at a dose of 2, 30, or 40 J/m<sup>2</sup>, and then incubated in fresh medium for the indicated times. Where indicated, they were incubated with 100 nm pifithrin- $\alpha$  (Calbiochem) for 3 h before and after UV irradiation.

**Cell Cycle Analysis**—Cells were collected by exposure to trypsin, washed with PBS, fixed in ice-cold 70% ethanol, and stored at -20 °C. They were subsequently washed twice with PBS, incubated for 30 min at room temperature with RNase A (100  $\mu$ g/ml), and stained with PI (25  $\mu$ g/ml) for 30 min. Flow cytometry was performed with a FACSCalibur instrument, and data were analyzed with CellQuest software (BD Biosciences). For assay of BrdUrd incorporation, cells were labeled with 10  $\mu$ M BrdUrd, fixed, denatured for 30 min at room temperature with PBS containing 2 N HCl, and washed with PBS. They were then incubated for 30 min with a 1:1 dilution of a fluorescein-conjugated mouse mAb to BrdUrd (BD Biosciences), counterstained with PI, and analyzed by flow cytometry.

**Measurement of Apoptosis**—Apoptosis was evaluated by flow cytometric determination of the fraction of cells with a sub-G<sub>1</sub> DNA content as well as by detection of DNA fragmentation with the use of a cell death detection enzyme-linked immunosorbent assay plus assay kit (Roche Applied Science). For detection of cells in the early stage of apoptosis, cells were fixed with 70% ethanol, stained for 60 min at room temperature with a 1:100 dilution of the M30 CytoDeath antibody (Roche Applied Science), which recognizes a specific caspase cleavage product of cytokeratin 18, in PBS containing 1% bovine serum albumin and 0.1% Tween 20, incubated for 30 min with fluorescein isothiocyanate-conjugated secondary antibodies, counterstained with PI, and analyzed by flow cytometry.

**Immunoblot Analysis**—Details of immunoblot analysis and various antibodies used are described in the supplemental Materials and Methods.

**Immunofluorescence Analysis**—Cells were washed with TBS, incubated for 2 min at room temperature with 3.7% formaldehyde, washed again with TBS, and fixed with 80% methanol for 5 min at room temperature. They were then stained with the following primary antibodies for 1 h at room temperature: mouse mAb DO-1 to p53 (1:1000 dilution); rabbit polyclonal antibodies to p53 (1:100 dilution; FL393, Santa Cruz Biotechnology); rabbit polyclonal antibodies to pol II LS (N-20, 1:500 dilution); and rabbit polyclonal antibodies to  $\gamma$ -H2AX (1:100 dilution; Trevigen). After washing with TBS, the cells were incu-

bated with fluorescein isothiocyanate-conjugated secondary antibodies, counterstained with PI, mounted with 1,4-diazabicyclo-(2,2,2)-octane-glycerol, and observed with a confocal laser-scanning microscope (Fluoview, Olympus) Mitochondria were stained by MitoTracker Red (Molecular Probes). For staining of BrdUrd, cells were labeled with 10  $\mu$ M BrdUrd for 24 h before harvesting, fixed, denatured for 15 min with 2 N HCl, washed with TBS, and incubated with a fluorescein-conjugated mouse mAb to BrdUrd.

**Semiquantitative RT-PCR Analysis**—Total RNA was isolated from cells with the use of an RNeasy spin column kit (Qiagen), and 2.5  $\mu$ g were converted to cDNA with SuperScript II reverse transcriptase (Invitrogen). The exponential phase of PCR was determined from 22 to 30 cycles to allow semiquantitative comparisons among cDNA samples subjected to identical reactions. Each PCR protocol included an initial denaturation at 95 °C for 5 min followed by 30 cycles (*pol II LS*), 25 cycles (*p53*, *p21*<sup>Cip1</sup>, *Bax*, *GADD45*, *p53DINP1 $\alpha$* ), or 22 cycles (*Noxa* and *GAPDH*) of 95 °C for 1 min, 55–60 °C for 1 min, and 72 °C for 1 min and was performed with a GeneAmp PCR system 9700 (PerkinElmer Life Sciences). The PCR products were separated by electrophoresis on 2% agarose gels.

**Adenovirus Infection**—The four replication-deficient recombinant adenoviral vectors used in the present study were described previously (22, 23). Ad5CMV-p53, Ad5CMV-p21<sup>Cip1</sup>, and Ad5CMV-p27<sup>Kip1</sup> encode wild-type human p53, p21<sup>Cip1</sup>, and p27<sup>Kip1</sup>, respectively, under the control of the human cytomegalovirus promoter. The Ad5CMV-Luc vector encoding luciferase was used as a control. Adenoviruses were propagated and titrated in the permissive 293 cell line. Viral infection titers were determined as described previously (24). Cells ( $3 \times 10^6$ ) were plated 24 h before infection and cultured for the indicated times with each adenovirus at a multiplicity of infection of 100 plaque-forming units per cell.

**Subcellular Fractionation**—Adherent and floating cells ( $2 \times 10^7$ ) were harvested and washed with PBS, and mitochondrial and cytosolic fractions were isolated with the use of an Optiprep Mitochondria Isolation Kit (Pierce, reagent-based method). The mitochondrial pellet was lysed in SDS sample buffer, and the cytosolic supernatant was concentrated with a Microcon device (Millipore, size cut-off of 10 kDa) before analysis by SDS-PAGE and immunoblotting.

#### RESULTS

**Inhibition of pol II-dependent Transcription Induces p53-mediated Apoptosis**—To evaluate the role of p53 in transcriptional blockade-mediated cell death, we treated p53 knock-out HCT116 cells (25) versus their parental control cells with  $\alpha$ -amanitin, a specific inhibitor of pol II-dependent transcription.  $\alpha$ -Amanitin binds directly to the LS of pol II (26) and specifically induces its ubiquitination and consequent degradation (27). Consistent with previous observations (28), cells treated with  $\alpha$ -amanitin manifested a time-dependent decrease in the total amount of mRNA and were unable to resume mRNA synthesis.<sup>2</sup> As shown in Fig. 1A, flow cytometry revealed that exposure of parental HCT116 (*p53*<sup>+/+</sup>) cells to  $\alpha$ -amanitin resulted in a substantial increase in the size of the sub-G<sub>1</sub> (apoptotic) cell population. However, knock-out of p53 (*p53*<sup>-/-</sup>) markedly reduced the sensitivity of HCT116 cells to death induced by  $\alpha$ -amanitin, indicating that the apoptosis elicited by these pol II inhibitors is p53-dependent.

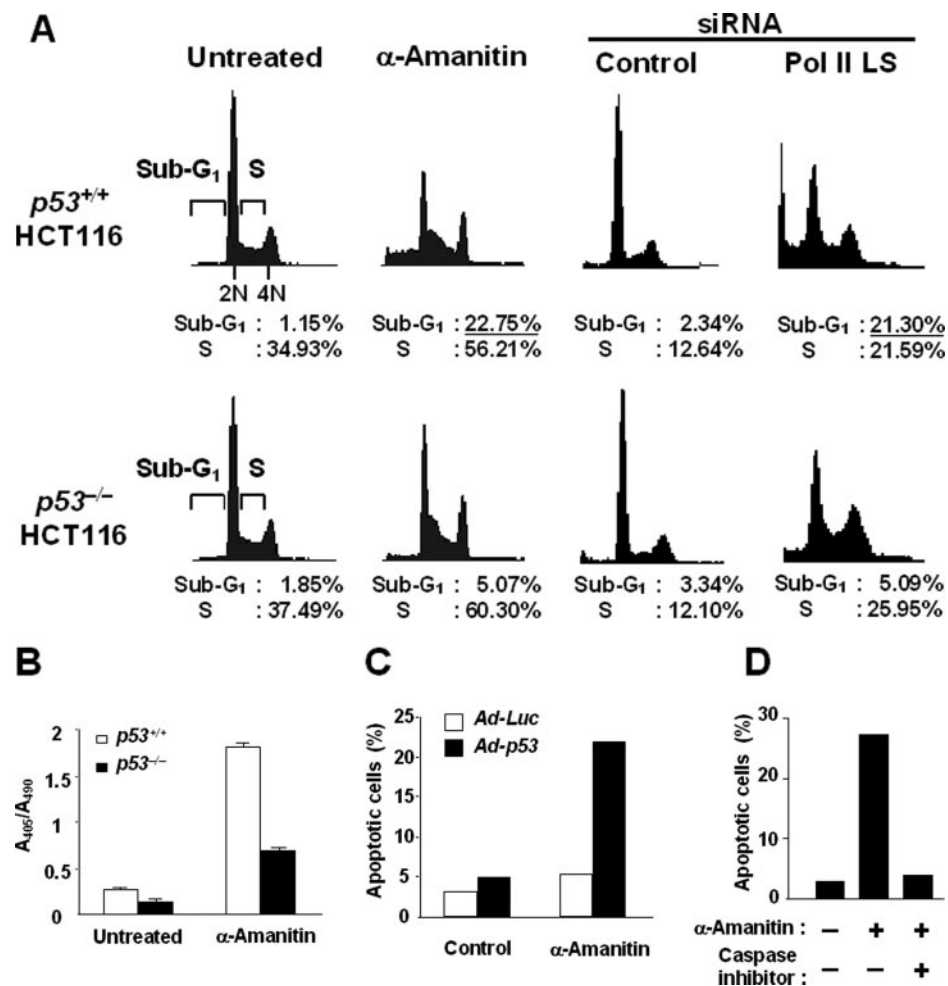
To validate further these results, we used RNA interference (RNAi) to abrogate expression of the pol II large subunit, which is an alternative approach to inhibit pol II-dependent transcription. Transfection of cells with small interfering RNAs (siRNA) specific for the pol II large subunit resulted in reduction in levels of the protein (supplemental Fig. S1). Similar to the data obtained by  $\alpha$ -amanitin, depletion of pol II significantly induced apoptosis in *p53*<sup>+/+</sup> HCT116 cells but not in *p53*<sup>-/-</sup> HCT116 cells (Fig. 1A).

To confirm whether  $\alpha$ -amanitin induces cell death by activation of apoptotic pathways, we performed an enzyme-linked immunosorbent-based assay that measures the level of cytoplasmic nucleosomal DNA fragments (Fig. 1B). Consistent with

<sup>2</sup> Y. Arima and H. Saya, unpublished data.

**FIG. 1. Inhibition of pol II-dependent transcript induces p53- and caspase-dependent apoptosis in HCT116 cells.**

**A**, induction of p53-dependent apoptosis in HCT116 human colon carcinoma cells. Both  $p53^{+/+}$  and  $p53^{-/-}$  HCT116 cells were incubated with  $\alpha$ -amanitin (10  $\mu$ g/ml) for 24 h or pol II LS siRNA for 48 h, after which the extent of apoptosis was determined by flow cytometry. **B**,  $\alpha$ -amanitin induces DNA fragmentation in  $p53^{+/+}$  HCT116 cells. HCT116 cells ( $p53^{+/+}$  or  $p53^{-/-}$ ) were incubated with or without  $\alpha$ -amanitin (10  $\mu$ g/ml) for 24 h, after which the amount of cytoplasmic histone-associated DNA fragments was determined with a quantitative sandwich enzyme-linked immunosorbent assay (substrate reaction time, 10 min). Data are presented as the 405/490 nm absorbance ratio and are means  $\pm$  S.D. of values from three independent experiments. **C**,  $\alpha$ -amanitin-induced apoptosis in  $p53^{-/-}$  HCT116 cells expressing exogenous p53. Cells were infected for 24 h with recombinant adenoviruses encoding luciferase (*Ad-Luc*) or p53 (*Ad-p53*) and then incubated in the absence or presence of  $\alpha$ -amanitin (10  $\mu$ g/ml) for 24 h. Treated cells were subjected to flow cytometric analysis for apoptosis. **D**, caspase dependence of  $\alpha$ -amanitin-induced apoptosis in HCT116 cells. Cells ( $p53^{+/+}$ ) were treated with  $\alpha$ -amanitin (10  $\mu$ g/ml) in the absence or presence of 20  $\mu$ M Z-Val-Ala-Asp(OMe)-fmk for 24 h, after which the extent of apoptosis was determined by flow cytometry.



the data obtained by flow cytometry,  $\alpha$ -amanitin induced a substantially larger increase in the amount of cytoplasmic DNA fragments in  $p53^{+/+}$  HCT116 cells than in the  $p53^{-/-}$  cells. Microscopic observation of HCT116 cells stained with PI also revealed that  $\alpha$ -amanitin induced a marked increase in the proportion of cells with fragmented chromatin (supplemental Fig. S2), a characteristic of apoptosis. Given that  $p53^{-/-}$  HCT116 cells are resistant to  $\alpha$ -amanitin-induced apoptosis (Fig. 1, A and B), we next examined whether restoration of p53 expression in these cells renders them sensitive to this effect of  $\alpha$ -amanitin. Adenovirus-mediated expression of p53, but not that of luciferase, indeed restored the sensitivity of  $p53^{-/-}$  cells to  $\alpha$ -amanitin-induced apoptosis (Fig. 1C and supplemental Fig. S3), supporting the notion that p53 expression is required for this effect of  $\alpha$ -amanitin. In addition, the apoptosis induced by  $\alpha$ -amanitin was completely blocked by the presence of the caspase inhibitor Z-Val-Ala-Asp(OMe)-fmk (Fig. 1D). These results indicate that inhibition of pol II-dependent transcription promotes p53- and caspase-dependent apoptosis.

**$\alpha$ -Amanitin Induces pol II Degradation and p53 Accumulation without DNA Double-strand Breaks**— $\alpha$ -Amanitin induces the ubiquitination of the C-terminal domain of the LS of pol II (29) and consequent proteasome-mediated protein degradation (27). We examined the effect of  $\alpha$ -amanitin on the abundance of the LS of pol II in  $p53^{+/+}$  or  $p53^{-/-}$  HCT116 cells by immunoblot (Fig. 2A) and immunofluorescence (supplemental Fig. S4) analyses. The toxin induced degradation of pol II LS in both types of HCT116 cells, suggesting that p53 does not contribute to  $\alpha$ -amanitin-induced pol II degradation.

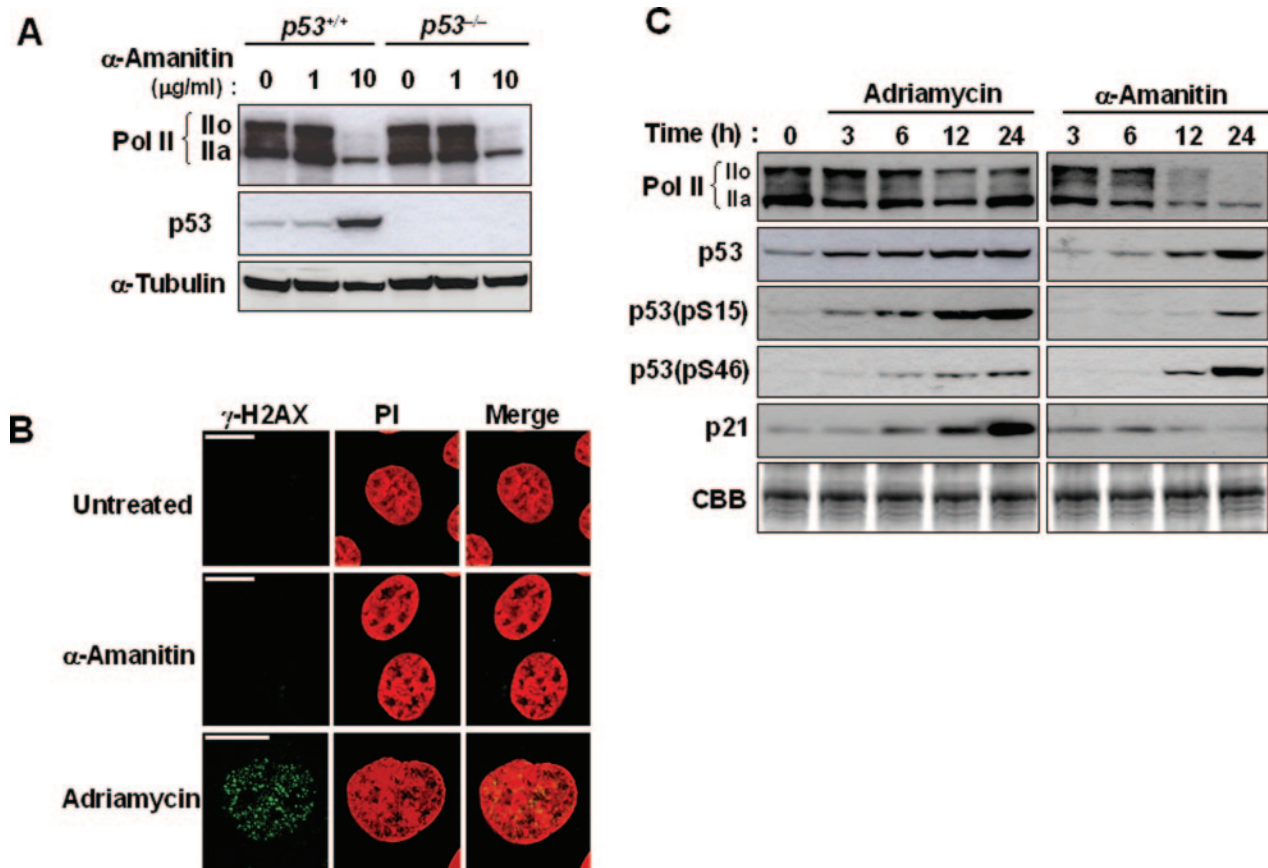
Given that apoptosis induced by  $\alpha$ -amanitin was shown to be p53-dependent, we next examined the effect of the drug on the

amount of p53. Consistent with previous observations (19, 28, 30), immunoblot analysis revealed that  $\alpha$ -amanitin elicited the accumulation of p53 in  $p53^{+/+}$  HCT116 cells (Fig. 2A). Accumulation of p53 by inhibition of pol II was also confirmed by depletion of pol II large subunit using siRNA (supplemental Fig. S1).

To verify that the p53 accumulation elicited by  $\alpha$ -amanitin was not attributable to the induction of DNA damage, we checked for the presence of the  $\gamma$ -H2AX complex, which can be a marker of DNA double-strand breaks (DSBs) (31, 32), in  $\alpha$ -amanitin-treated HCT116 cells by immunofluorescence analysis. To avoid the detection of DSBs attributable to apoptotic DNA fragmentation, we examined cells for the presence of  $\gamma$ -H2AX foci 18 h after exposure to drugs (Fig. 2B). Whereas adriamycin induced the formation of  $\gamma$ -H2AX foci,  $\alpha$ -amanitin did not, even though substantial p53 accumulation was apparent at this time point. These results suggest that the accumulation of p53 induced by  $\alpha$ -amanitin is not caused by the induction of DSBs.

The accumulation and activation of p53 are regulated by post-translational modifications, including protein phosphorylation and acetylation (33). Given that phosphorylation of p53 contributes to both the stabilization and activation of the protein, we examined the time course of p53 phosphorylation on Ser<sup>15</sup> and Ser<sup>46</sup> in  $p53^{+/+}$  HCT116 cells treated with adriamycin or  $\alpha$ -amanitin (Fig. 2C). Adriamycin-induced Ser<sup>15</sup> phosphorylation was apparent at 3 h, as was p53 accumulation; an increase in the extent of phosphorylation of Ser<sup>46</sup> was not apparent until 6 h, consistent with the delay in the phosphorylation of this residue compared with that of Ser<sup>15</sup> observed previously in adriamycin-treated cells (6). In contrast, an increase in the extent of Ser<sup>46</sup>

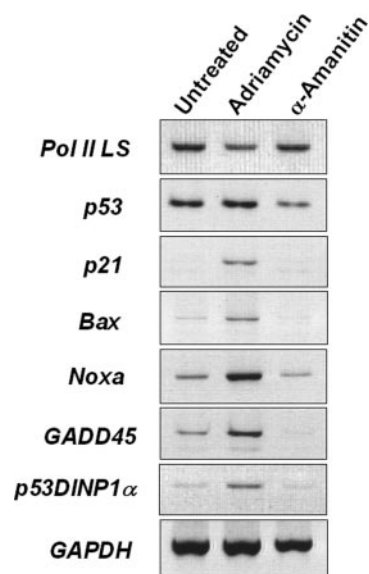




**FIG. 2. Induction of pol II LS degradation and of p53 phosphorylation and accumulation in HCT116 cells by  $\alpha$ -amanitin.** A,  $\alpha$ -amanitin-induced pol II LS degradation and p53 accumulation. Cells ( $p53^{+/+}$  or  $p53^{-/-}$ ) were incubated in the presence of the indicated concentrations of  $\alpha$ -amanitin for 24 h, after which cell lysates were subjected to immunoblot analysis with antibodies against pol II LS, p53 (DO-1), and  $\alpha$ -tubulin (loading control). *Ilo*, hyperphosphorylated form of pol II; *Ila*, hypophosphorylated form of pol II. B,  $\alpha$ -amanitin does not induce DNA double-strand breaks. Cells ( $p53^{+/+}$ ) were treated with  $\alpha$ -amanitin (10  $\mu$ g/ml) or adriamycin (500 ng/ml) for 18 h and then both stained with antibodies to  $\gamma$ -H2AX (green) and counterstained with PI (red). The merged images are shown in the right panels. Scale bars, 20  $\mu$ m. C, phosphorylation and accumulation of p53 induced by adriamycin or  $\alpha$ -amanitin. Cells ( $p53^{+/+}$ ) were treated with adriamycin (500 ng/ml) or  $\alpha$ -amanitin (10  $\mu$ g/ml) for the indicated times, after which cell lysates were subjected to immunoblot analysis with antibodies to pol II LS, to p53 (DO-1), to p53 phosphorylated on Ser<sup>15</sup>, and to p53 phosphorylated on Ser<sup>46</sup> and to p21<sup>Cip1</sup>. The membrane was stained with Coomassie Brilliant Blue (CBB) to show equal loading of the samples.

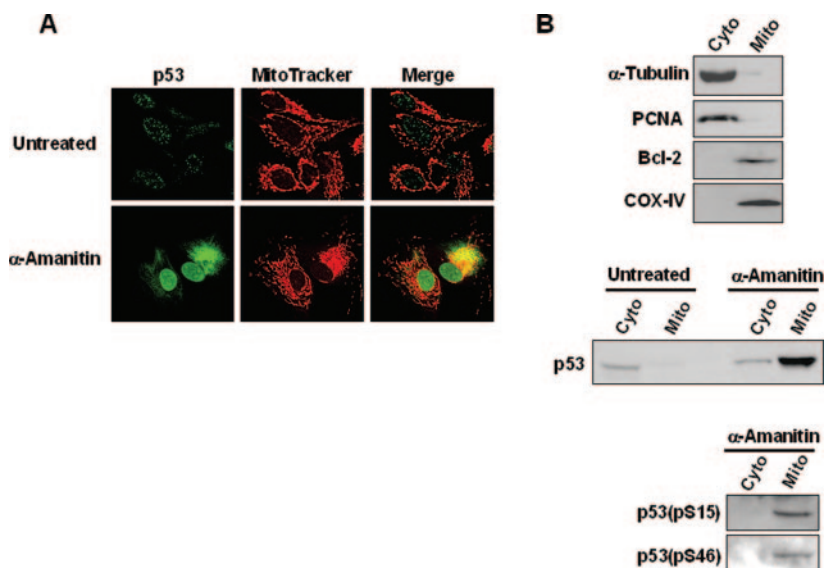
phosphorylation was apparent at 12 h, coincident with the onset of p53 accumulation, and Ser<sup>15</sup> phosphorylation was not detected until 24 h in cells treated with  $\alpha$ -amanitin. These results thus suggest that  $\alpha$ -amanitin triggers a slower p53 response in comparison to adriamycin and may activate a signaling pathway that differs from that responsible for the p53 stabilization in response to DNA damage.

**Transcription of p53 Target Genes Is Blocked in  $\alpha$ -Amanitin-treated Cells**— $\alpha$ -Amanitin blocks pol II-dependent gene transcription. Therefore, we next confirmed that p53-mediated apoptosis induced by  $\alpha$ -amanitin is independent of the transactivation activity of p53. With the use of semiquantitative RT-PCR analysis, we determined the amounts of mRNAs derived from various p53 target genes, including those for p21<sup>Cip1</sup>, Bax, Noxa, GADD45 (34), and p53DINP1 $\alpha$  (35), in HCT116 cells treated with adriamycin or  $\alpha$ -amanitin for 24 h (Fig. 3). Whereas adriamycin increased the abundance of the mRNAs for all p53 target genes examined,  $\alpha$ -amanitin did not. The abundance of p53 mRNA was reduced by treatment of cells with  $\alpha$ -amanitin but not by exposure to adriamycin. Additionally, our time course experiments showed that the expression of these p53 target genes was not up-regulated in  $\alpha$ -amanitin-treated cells throughout the treatment period (data not shown). These data suggest that transcriptional blockade triggers a p53-dependent apoptosis that is not dependent upon p53-mediated changes in gene expression.



**FIG. 3. Inhibition by  $\alpha$ -amanitin of transcription of 53 target genes.** HCT116 cells were incubated for 24 h in the absence or presence of adriamycin (500 ng/ml) or  $\alpha$ -amanitin (10  $\mu$ g/ml), after which the abundance of mRNAs for the indicated proteins was determined by semiquantitative RT-PCR analysis. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

**FIG. 4. Translocation of p53 to mitochondria in response to transcriptional blockade.** *A*, immunofluorescence localization of p53 to mitochondria in  $\alpha$ -amanitin-treated cells. MRC-5 cells were incubated in the absence or presence of  $\alpha$ -amanitin (10  $\mu$ g/ml) for 48 h and then stained with antibodies to p53 (FL393) (green) and MitoTracker (red). Merged images are shown in the right panels. *B*, enrichment of p53 in the mitochondrial fraction of cells treated with  $\alpha$ -amanitin. Mitochondrial (Mito) and cytosolic (Cyto) fractions prepared from MRC-5 cells were subjected to immunoblot analysis with antibodies to the cytosolic proteins  $\alpha$ -tubulin and PCNA and to the mitochondrial proteins Bcl-2 and cytochrome oxidase IV (COX-IV) (upper panels). Mitochondrial and cytosolic fractions of MRC-5 cells were also subjected to immunoblot analysis with antibodies to p53 (DO-1) or to p53 phosphorylated on Ser<sup>15</sup> or Ser<sup>46</sup> after the cells were exposed to  $\alpha$ -amanitin for 48 h (middle and lower panels).



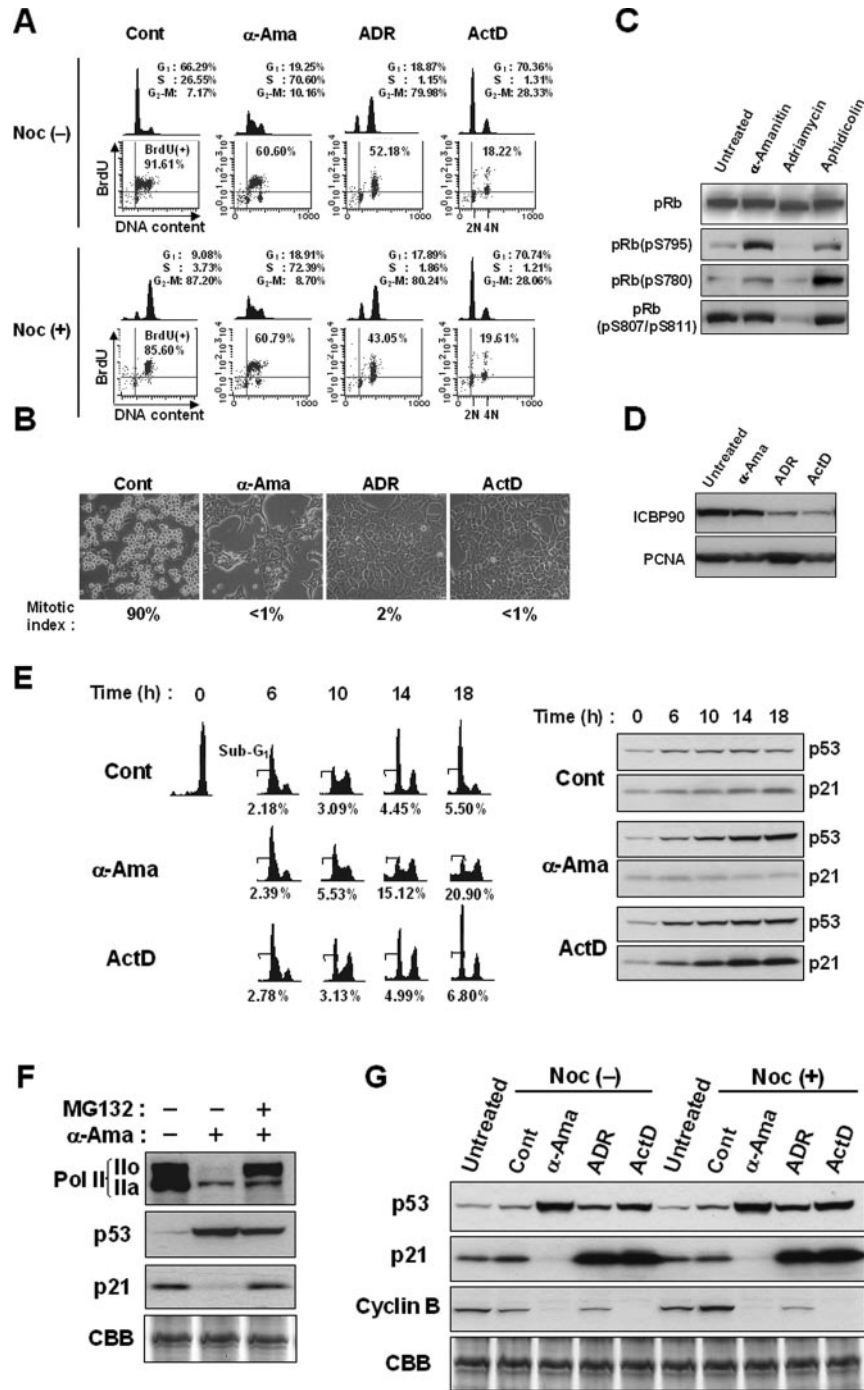
*Translocation of p53 to Mitochondria in  $\alpha$ -Amanitin-treated Cells*—Recent studies have suggested that p53-dependent apoptosis can occur in the absence of gene transcription (12, 13) and that a fraction of p53 molecules that accumulate in response to DNA damage translocates to mitochondria and thereby promotes apoptosis (14, 15). We therefore examined whether p53 molecules that accumulate in response to transcriptional blockade translocate to mitochondria. Confocal microscopic analysis was carried out by using MRC-5 human normal fibroblasts that have wild-type p53. MRC-5 cells are suitable for investigating the subcellular distribution of endogenous p53 because the cells have flat and large morphology and can be easily observed cytoplasmic structures. We also confirmed that  $\alpha$ -amanitin elicits p53 accumulation and apoptosis in MRC-5 cells (supplemental Fig. S5, A and B) as observed in HCT116 cells. Although p53 protein was detectable at a low level in the nuclei of untreated cells, it was present in increased amounts in  $\alpha$ -amanitin-treated cells and was localized to mitochondria in a substantial proportion of these cells (Fig. 4A). Cell fractionation experiments also demonstrate that p53 accumulated by  $\alpha$ -amanitin treatment is primarily detected in mitochondrial fractions, and those mitochondrial p53 molecules are phosphorylated on Ser<sup>15</sup> and/or Ser<sup>46</sup> (Fig. 4B). These findings thus suggest that p53 molecules that accumulate in response to transcriptional blockade translocate to the mitochondria, which may activate the apoptotic program.

*$\alpha$ -Amanitin Treatment Does Not Induce G<sub>1</sub> Arrest and Allows S Phase Entry*— $\alpha$ -Amanitin increased the size of the cell population in S phase in a p53-independent manner (Fig. 1A). Depletion of pol II LS by siRNA also increased the population of S phase cells in comparison to control siRNA treatment (Fig. 1A). Therefore, to investigate further the effect of  $\alpha$ -amanitin on cell cycle progression, HCT116 cells were first incubated with  $\alpha$ -amanitin or other reagents alone and then in the absence or presence of nocodazole, which induces cell cycle arrest in prometaphase through activation of the spindle assembly checkpoint. Consistent with previous observations (36), treatment of the cells with actinomycin D, which is an RNA polymerase I inhibitor, or adriamycin resulted in G<sub>1</sub> or G<sub>2</sub> arrest, respectively (Fig. 5A). In contrast, cells treated with  $\alpha$ -amanitin incorporated BrdUrd and accumulated predominantly in the S phase (~61%); they neither arrested in G<sub>1</sub> nor entered M phase regardless of whether or not they were exposed to nocodazole (Fig. 5, A and B). Whereas the retinoblastoma protein (pRb) was dephosphorylated in cells treated with adriamycin, it

was highly phosphorylated in  $\alpha$ -amanitin-treated cells (Fig. 5C) as it was in cells exposed to a DNA polymerase  $\alpha$  inhibitor aphidicolin, which induced S phase arrest. Furthermore, the abundance of ICBP90, a nuclear protein whose expression is down-regulated during DNA damage-induced G<sub>1</sub> arrest (20), was not affected by treatment of HCT116 cells with  $\alpha$ -amanitin, whereas it was decreased by treatment with adriamycin or actinomycin D (Fig. 5D). Together, these findings suggest that cells exposed to  $\alpha$ -amanitin progress through the G<sub>1</sub>-S transition and arrest in S phase.

We next investigated the mechanism by which  $\alpha$ -amanitin-treated cells are able to enter S phase even though they manifest accumulation of p53. DNA damage is known to induce expression of the cyclin-dependent kinase (CDK) inhibitor p21<sup>Cip1</sup> via p53-dependent transactivation (25). p21<sup>Cip1</sup> binds to and inhibits the activity of both PCNA and the complexes of CDK2 with cyclin A or cyclin E, thereby blocking progression of cells from G<sub>1</sub> to S (37). Given that the levels of p21<sup>Cip1</sup> protein and mRNA were down-regulated in both the  $\alpha$ -amanitin-treated cells and the siRNA-mediated pol II-depleted cells (Figs. 2C and 3 and supplemental Fig. S1), cells might be able to enter S phase despite p53 accumulation. To address this issue further, we monitored changes in the levels of p21<sup>Cip1</sup> during cell cycle progression of the  $\alpha$ -amanitin-treated cells (Fig. 5E). HCT116 cells were synchronized at metaphase by nocodazole treatment and then released into fresh medium containing  $\alpha$ -amanitin or actinomycin D, the latter of which stabilizes p53, up-regulates p21<sup>Cip1</sup> expression, and induces G<sub>1</sub> arrest. The size of the G<sub>1</sub> population was markedly reduced and that of the sub-G<sub>1</sub> (apoptotic) population was increased for cells treated with  $\alpha$ -amanitin compared with the corresponding populations of cells treated with actinomycin D or those left untreated. Immunoblot analysis revealed that the amount of p21<sup>Cip1</sup> protein was reduced in cells exposed to  $\alpha$ -amanitin, although it was markedly increased in response to actinomycin D (Fig. 5E).

The abundance of p21<sup>Cip1</sup> is regulated not only by transcriptional induction but also by ubiquitin-mediated proteasomal degradation (38). To examine whether the  $\alpha$ -amanitin-induced down-regulation of p21<sup>Cip1</sup> expression was attributable to protein degradation by the ubiquitin-proteasome pathway, we treated HCT116 cells with  $\alpha$ -amanitin in the presence of MG132, a proteasome inhibitor. The decrease in the amount of p21<sup>Cip1</sup> induced by  $\alpha$ -amanitin was prevented by MG132 (Fig. 5F), indicating that p21<sup>Cip1</sup> undergoes ubiquitin-mediated pro-



**FIG. 5. Treatment with  $\alpha$ -amanitin induces G<sub>1</sub>-S transition and S phase accumulation in HCT116 cells.** *A*, entry of  $\alpha$ -amanitin-treated cells into S phase. Cells were incubated in the absence (*Cont*) or presence of  $\alpha$ -amanitin (10  $\mu$ g/ml,  $\alpha$ -Ama), adriamycin (500 ng/ml, *ADR*), or actinomycin D (20 nM, *ActD*) for 18 h and then in the presence of 10  $\mu$ M BrdUrd for an additional 5 h. They were then subjected to flow cytometric analysis of cell cycle status (*upper panels*). In parallel incubations, nocodazole (100 ng/ml, *Noc*) was added to the culture medium 12 h before analysis to induce metaphase arrest (*lower panels*). *B*, inhibition of mitotic entry by  $\alpha$ -amanitin. Cells were incubated in the absence or presence of  $\alpha$ -amanitin, adriamycin, or actinomycin D for 24 h and then in the additional presence of nocodazole for 12 h. The cells were then examined by phase-contrast microscopy, and the percentage of mitotic cells was determined by visual inspection of a total of 200 cells per condition. *C*, phosphorylation of pRb in  $\alpha$ -amanitin-treated cells. Cells were incubated in the absence or presence of  $\alpha$ -amanitin, adriamycin, or aphidicolin (1  $\mu$ g/ml) for 24 h, after which cell lysates were subjected to immunoblot analysis with antibodies to pRb or to pRb phosphorylated on serines 795, 780 or 807, and 811. *D*, ICBP90 expression in  $\alpha$ -amanitin-treated cells. Cells were incubated in the absence or presence of  $\alpha$ -amanitin, adriamycin, or actinomycin D for 36 h, after which cell lysates were subjected to immunoblot analysis with antibodies to ICBP90. Proliferating-cell nuclear antigen was used as a loading control because the abundance of PCNA is not affected by any of these three agents. *E*, down-regulation of p21<sup>Cip1</sup> in  $\alpha$ -amanitin-treated cells. Cells were synchronized in metaphase by nocodazole treatment and then released into fresh medium in the absence or presence of  $\alpha$ -amanitin or actinomycin D for the indicated times. They were then subjected to flow cytometric analysis of DNA content (*left panels*) or to immunoblot analysis with antibodies to p53 (DO-1) or to p21<sup>Cip1</sup> (*right panels*). *F*, degradation of p21<sup>Cip1</sup> by the ubiquitin-proteasome pathway in  $\alpha$ -amanitin-treated cells. Cells were incubated with or without  $\alpha$ -amanitin for 12 h and then in the additional absence or presence of 10  $\mu$ M MG132 for 12 h, after which cell lysates were subjected to immunoblot analysis with antibodies indicated. The membrane was stained with Coomassie Brilliant Blue (CBB) to show equal loading of the samples. *G*, down-regulation of cyclin B in  $\alpha$ -amanitin-treated cells. Cells were incubated in the absence or presence of  $\alpha$ -amanitin, adriamycin, or actinomycin D for 24 h and then in the absence or presence of nocodazole for 12 h. Cell lysates were then subjected to immunoblot analysis of p53, p21<sup>Cip1</sup>, and cyclin B. Untreated cells were similarly analyzed. The membrane was stained with Coomassie Brilliant Blue to show equal loading of the samples.



teolysis in response to  $\alpha$ -amanitin. Thus, both transcriptional suppression and protein degradation appear to prevent the accumulation of p21<sup>Cip1</sup> in  $\alpha$ -amanitin-treated cells and thereby lead to S phase entry.

Activation of the cyclin B-Cdc2 complex is a key event in the transition from G<sub>2</sub> to M phase (39). To investigate the mechanism of S phase arrest in  $\alpha$ -amanitin-treated cells, we assessed the level of cyclin B in HCT116 cells (Fig. 5G). Immunoblot analysis revealed that cyclin B was not detectable in cells treated with  $\alpha$ -amanitin, suggesting that the cells are prevented from entering mitosis as a result of down-regulation of this cyclin.

**$\alpha$ -Amanitin-treated Cells Undergo Apoptosis on Entry into S Phase**—Nocodazole synchronization revealed that  $\alpha$ -amanitin induced apoptosis ~14 h after the release of cells from metaphase arrest (Fig. 5E), a time that coincides approximately with the G<sub>1</sub>-S transition. We therefore investigated whether cells treated with  $\alpha$ -amanitin undergo apoptosis as they enter S phase. HCT116 cells were synchronized at G<sub>0</sub>-G<sub>1</sub> by serum deprivation and then re-exposed to serum in the absence or presence of  $\alpha$ -amanitin (Fig. 6A). Cells treated with  $\alpha$ -amanitin exhibited a marked delay in progression through S phase compared with control cells. In addition, treatment with  $\alpha$ -amanitin resulted in a large increase in the number of cells positive for M30 CytoDeath antibody, which recognizes a specific caspase cleavage product of cytokeratin 18 and can be a marker of early apoptosis, at the time (24 h after release) when the cells began to accumulate in S phase (Fig. 6A). Furthermore, this apoptotic marker was detected in the  $\alpha$ -amanitin-treated cells during early S phase but not at G<sub>2</sub>-M phase, suggesting that  $\alpha$ -amanitin induces p53-dependent apoptosis as cells enter S phase. This notion is further supported by the immunocytochemical finding that  $\alpha$ -amanitin-treated cells in which p53 was localized to mitochondria preferentially incorporate BrdUrd, indicating that they had entered S phase (Fig. 6B).

We next tested the possibility that induction of G<sub>1</sub> arrest is able to block  $\alpha$ -amanitin-induced apoptosis in HCT116 cells. The plant amino acid mimosine blocks the cell cycle in late G<sub>1</sub> phase by inducing the up-regulation of the CDK inhibitor p27<sup>Kip1</sup> at both transcriptional and post-transcriptional levels (40). Mimosine markedly inhibited the  $\alpha$ -amanitin-induced increase in the size of the sub-G<sub>1</sub> (apoptotic) cell population (Fig. 6C). Adenovirus-mediated overexpression of the CDK inhibitors p21<sup>Cip1</sup> or p27<sup>Kip1</sup> also induced cell cycle arrest at G<sub>1</sub> phase and prevented  $\alpha$ -amanitin-induced apoptosis (Fig. 6D). Specifically, these results suggest that S phase entry is required for the apoptosis induced by transcriptional blockade.

**Accumulation of p53 and Down-regulation of p21<sup>Cip1</sup> Lead to Apoptosis**—We then examined whether down-regulation of p21<sup>Cip1</sup> is also required for the induction of apoptosis by  $\alpha$ -amanitin. Treatment of p21<sup>-/-</sup> HCT116 cells with  $\alpha$ -amanitin resulted in p53 accumulation and massive apoptosis (Fig. 7A). This finding raised the hypothesis that p53 accumulation without p21<sup>Cip1</sup> induction triggers the apoptosis. We thus constructed this condition by adenovirus-mediated overexpression of p53 in the p21<sup>-/-</sup> cells, and we found that this combination induces massive apoptosis without  $\alpha$ -amanitin treatment (Fig. 7B). Furthermore, pifithrin- $\alpha$ , a specific inhibitor of p53-mediated transcriptional activation (41), did not inhibit the induction of apoptosis in p21<sup>-/-</sup> HCT116 cells by overexpression of p53 (Fig. 7B), suggesting that the transactivation activity of p53 does not contribute to this effect. These findings suggest the possibility that the p53-mediated transactivation-independent apoptosis is triggered by the accumulation of p53 without p21<sup>Cip1</sup> induction.

To verify further that transcriptional blockade induces p53-

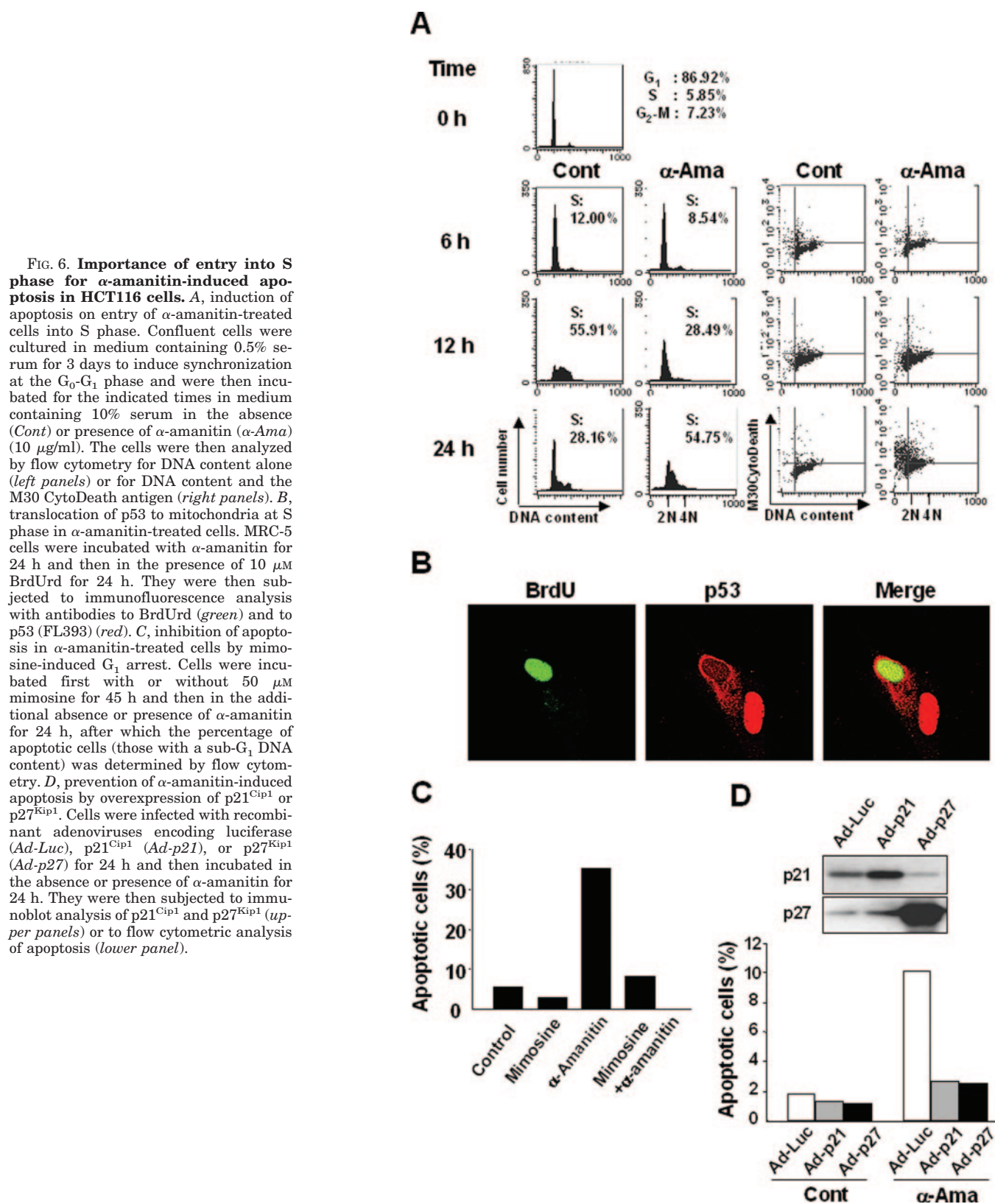
mediated transcription-independent apoptosis, we used cells expressing a transactivation-deficient p53. We established p53<sup>-/-</sup> HCT116 cells stably expressing wild-type p53 or a truncated form of p53-( $\Delta$ 1–39) (supplemental Fig. S6). p53-( $\Delta$ 1–39) lacks an N-terminal transactivation domain (residues 1–39), which is thus unable to transactivate p21<sup>Cip1</sup> expression (42). Treatment with  $\alpha$ -amanitin induced apoptosis in cells not only expressing wild-type p53 but also in cells expressing p53-( $\Delta$ 1–39), although the p53<sup>-/-</sup> HCT116 cells were insensitive to  $\alpha$ -amanitin treatment (Fig. 7C). These results thus support our hypothesis that apoptosis induced by transcriptional blockade is p53-dependent but transactivation-independent.

**High Dose UV Irradiation Induces Transcription-independent p53-mediated Apoptosis**—Here we showed that inhibition of pol II by  $\alpha$ -amanitin treatment or siRNA approach resulted in the p53 accumulation without p21<sup>Cip1</sup> induction, leading to apoptosis. We wanted to find out whether there is any biological condition inducing this type of cell death. As reported previously (16), exposure of HCT116 cells to a high dose of UV radiation (30 J/m<sup>2</sup>) induced the ubiquitin-mediated degradation of pol II LS, resulting in transcriptional blockade and apoptosis 24 h after the treatment (Fig. 7D, right panel). This treatment actually induced p53 accumulation and p21<sup>Cip1</sup> down-regulation (Fig. 7D, left panel), biochemical effects identical to those of  $\alpha$ -amanitin and pol II LS siRNA. In addition, the high dose UV-induced p53 was predominantly detected in the mitochondrial fraction (Fig. 7E). Together, these findings thus suggest that accumulation of p53 in mitochondria with down-regulation of p21<sup>Cip1</sup>, which resulted from transcriptional blockade, leads to apoptosis in high dose UV-irradiated cells.

## DISCUSSION

Blockade of pol II-dependent transcription has been shown previously to trigger a cell death signal (28). However, the mechanism that underlies such apoptosis has remained undefined. Furthermore, recent observations have revealed that p53 can directly translocate to the mitochondria and induce apoptosis in a transactivation-independent manner (14, 15), but the mechanisms triggering this apoptosis have remained unclear. We now provide evidence that links these two events. Inhibition of pol II by  $\alpha$ -amanitin treatment, RNAi approach, and high dose UV irradiation induces p53 accumulation without induction of p21<sup>Cip1</sup>, entry of cells into S phase, and p53-dependent but transactivation-independent cell death mediated by the translocation of p53 to mitochondria. Our data thus yield new insight into the mechanism of cell death induced by transcriptional blockade.

**Inhibition of pol II by  $\alpha$ -Amanitin Induces p53 Accumulation and p53-dependent Apoptosis**—Apoptosis induced by  $\alpha$ -amanitin was markedly greater in extent in p53<sup>+/+</sup> HCT116 cells than in the corresponding p53<sup>-/-</sup> cells and was blocked in the presence of a caspase inhibitor, indicating that transcriptional blockade induces p53-dependent, caspase-mediated apoptosis. Furthermore, this notion was supported by the finding that the  $\alpha$ -amanitin-resistant p53<sup>-/-</sup> cells underwent apoptosis by expression of exogenous p53. The accumulation of p53 appeared to be attributable to inhibition of p53 degradation, given that the amount of p53 mRNA was actually reduced by  $\alpha$ -amanitin treatment. These findings are consistent with previous results (43), showing that the accumulation of p53 induced by inhibition of pol II-dependent transcription results from down-regulation of MDM2 gene transcription. Therefore, in addition to the MDM2 down-regulation-mediated pathway, signaling triggered by modification of p53 protein may contribute to p53 accumulation. In fact, we also found that  $\alpha$ -amanitin induces p53 phosphorylation. The phosphorylation of p53, which is mediated by various stress-activated kinases (44), contributes



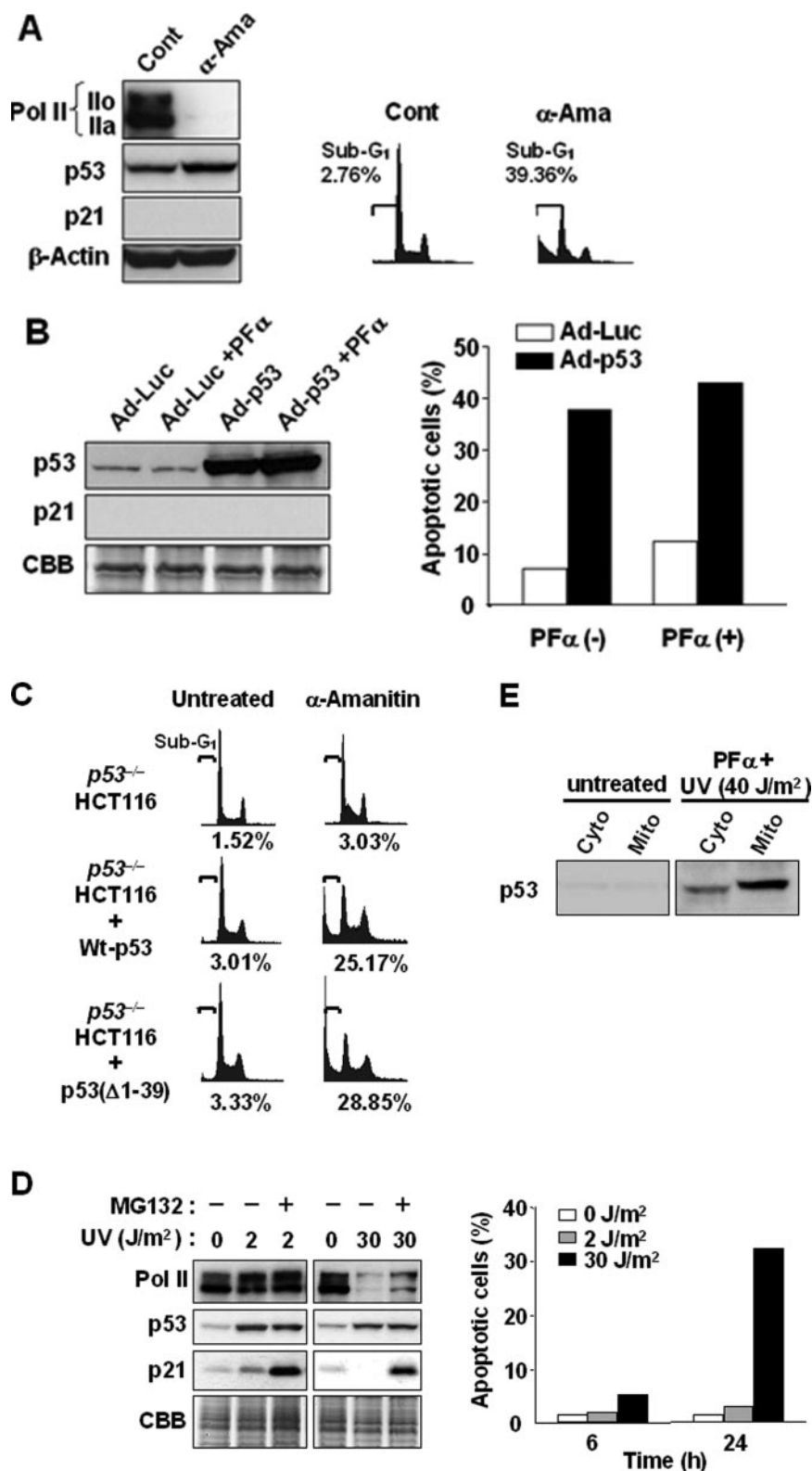
**FIG. 6. Importance of entry into S phase for  $\alpha$ -amanitin-induced apoptosis in HCT116 cells.** A, induction of apoptosis on entry of  $\alpha$ -amanitin-treated cells into S phase. Confluent cells were cultured in medium containing 0.5% serum for 3 days to induce synchronization at the  $G_0$ - $G_1$  phase and were then incubated for the indicated times in medium containing 10% serum in the absence (Cont) or presence of  $\alpha$ -amanitin ( $\alpha$ -Ama) (10  $\mu$ g/ml). The cells were then analyzed by flow cytometry for DNA content alone (left panels) or for DNA content and the M30 CytoDeath antigen (right panels). B, translocation of p53 to mitochondria at S phase in  $\alpha$ -amanitin-treated cells. MRC-5 cells were incubated with  $\alpha$ -amanitin for 24 h and then in the presence of 10  $\mu$ M BrdUrd for 24 h. They were then subjected to immunofluorescence analysis with antibodies to BrdUrd (green) and to p53 (FL393) (red). C, inhibition of apoptosis in  $\alpha$ -amanitin-treated cells by mimosine-induced  $G_1$  arrest. Cells were incubated first with or without 50  $\mu$ M mimosine for 45 h and then in the additional absence or presence of  $\alpha$ -amanitin for 24 h, after which the percentage of apoptotic cells (those with a sub- $G_1$  DNA content) was determined by flow cytometry. D, prevention of  $\alpha$ -amanitin-induced apoptosis by overexpression of p21<sup>Cip1</sup> or p27<sup>Kip1</sup>. Cells were infected with recombinant adenoviruses encoding luciferase (Ad-Luc), p21<sup>Cip1</sup> (Ad-p21), or p27<sup>Kip1</sup> (Ad-p27) for 24 h and then incubated in the absence or presence of  $\alpha$ -amanitin for 24 h. They were then subjected to immunoblot analysis of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> (upper panels) or to flow cytometric analysis of apoptosis (lower panel).

to the stabilization and activation of the protein. The DNA-damaging agent adriamycin induced the phosphorylation of p53 on Ser<sup>15</sup>, which is reportedly mediated by the kinase ataxia telangiectasia-mutated (45) and results in p53 accumulation, before that on Ser<sup>46</sup>. In contrast,  $\alpha$ -amanitin induced p53 phosphorylation on Ser<sup>46</sup> before the phosphorylation of p53 on Ser<sup>15</sup>, and the former reaction coincided with the accumulation

of p53. Transcriptional blockade by  $\alpha$ -amanitin might thus result in the activation of stress-activated kinases that are distinct from those activated by DNA-damaging agents and that phosphorylate and thereby stabilize p53. Indeed, caffeine, an inhibitor of ataxia telangiectasia-mutated (ATM) kinase and ATM and Rad3-related (ATR) kinase, did not inhibit  $\alpha$ -amanitin-induced p53 phosphorylation and accumulation,<sup>2</sup>



**FIG. 7. Apoptosis triggered by accumulation of p53 and down-regulation of p21<sup>Cip1</sup>.** *A*, enhanced  $\alpha$ -amanitin-induced apoptosis in HCT116 cells lacking p21<sup>Cip1</sup>. Cells ( $p21^{-/-}$ ) were incubated with or without (*Cont*)  $\alpha$ -amanitin ( $\alpha$ -*Ama*) for 24 h and then immunoblotted with antibodies against pol II LS, p53 (DO-1), p21<sup>Cip1</sup> and  $\beta$ -actin (loading control). Cells were also subjected to flow cytometric analyses for apoptosis. *B*, induction of apoptosis in  $p21^{-/-}$  HCT116 cells by overexpression of p53. Cells were incubated with or without 100 nM pifithrin- $\alpha$  (*PF $\alpha$* ) for 3 h and then infected with an adenoviral vector for luciferase or p53 for 24 h in the continued absence or presence of pifithrin- $\alpha$ . They were then subjected to immunoblot analysis of p53 (*left panel*) and to flow cytometric analysis of apoptosis (*right panel*). *CBB*, Coomassie Brilliant Blue. *C*,  $\alpha$ -Amanitin induces apoptosis in  $p53^{-/-}$  HCT116 cells expressing a transactivation-deficient p53.  $p53^{-/-}$  HCT116 cells and those stably expressing wild-type p53 or a truncated form of p53 (p53-( $\Delta$ 1-39)) were incubated with  $\alpha$ -amanitin (10  $\mu$ g/ml) for 24 h, after which the extent of apoptosis was determined by flow cytometry. *D*, accumulation of p53, down-regulation of p21<sup>Cip1</sup>, and apoptosis induced by high dose UV irradiation in HCT116 cells. Parental cells ( $p53^{+/+}$  and  $p21^{+/+}$ ) were exposed to UV radiation (0, 2, or 30  $J/m^2$ ) in the absence or presence of 10  $\mu$ M MG132. Cells harvested 6 h after irradiation were subjected to immunoblot analysis of pol II LS, p53, and p21<sup>Cip1</sup> (*left panels*), and those harvested 6 or 24 h after irradiation were subjected to flow cytometric analysis of apoptosis (*right panel*). *E*, accumulation of p53 in the mitochondrial fraction of cells exposed to UV radiation. Mitochondrial (*Mito*) and cytosolic (*Cyto*) fractions of MRC-5 cells were subjected to immunoblot analysis with antibodies to p53 (DO-1) 24 h after they were exposed to UV radiation (40  $J/m^2$ ) in the presence of pifithrin- $\alpha$ .



suggesting that these kinases do not contribute to the effects of  $\alpha$ -amanitin. We also found that transcriptional blockade did not activate the Chk1 (supplemental Fig. S7). Furthermore,  $\alpha$ -amanitin did not induce p53 accumulation in M059J cells (46), which are deficient in the catalytic subunit of DNA-dependent protein kinase (supplemental Fig. S8), suggesting a possible role for DNA-dependent protein kinases in the phosphorylation of p53 in response to transcriptional blockade in the absence of DNA damage.

*Cells Treated with  $\alpha$ -Amanitin Undergo Apoptosis on Entry into S Phase*—The accumulation of p53 in response to DNA damage results in the transcriptional activation of p53 target genes whose products either induce cell cycle arrest (such as p21<sup>Cip1</sup>) or trigger apoptosis. However, we found that the expression of p21<sup>Cip1</sup> was down-regulated by  $\alpha$ -amanitin as a result of both suppression of gene transcription and ubiquitin-dependent protein degradation. The  $\alpha$ -amanitin-treated cells were thus prevented from arresting at G<sub>1</sub> phase. In addition,

we found that the abundance of p27<sup>Kip1</sup>, another CDK2 inhibitor, was also down-regulated by  $\alpha$ -amanitin treatment in HCT116 and MRC-5 cells (supplemental Fig. S9). Thus, despite the fact that they accumulate p53, cells treated with  $\alpha$ -amanitin proceed to S phase, as confirmed by the status of pRb phosphorylation, flow cytometric analysis of DNA content, and BrdUrd incorporation. The down-regulation of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> might possibly be due to cleavage by the CPP32-like caspase during apoptosis (47). However, the abundance of p21<sup>Cip1</sup> in  $\alpha$ -amanitin-treated HCT116 cells was reduced prior to the appearance of the apoptotic phenotype (Fig. 2C). Moreover, Skp2, which is a subunit of the Skp1-Cullin-F-box protein ubiquitin ligase that controls the proteolysis of p27<sup>Kip1</sup> and p21<sup>Cip1</sup> (48–50), was up-regulated in  $\alpha$ -amanitin-treated HCT116 cells (supplemental Fig. S10), suggesting that signaling activated by transcriptional blockade results in the activation of Skp2, which then mediates the down-regulation of p21<sup>Cip1</sup> and p27<sup>Kip1</sup>.

Overexpression of exogenous p53 markedly enhanced cell death in p21<sup>-/-</sup> HCT116 cells (Fig. 7B), suggesting that the entry of cells with phosphorylated and accumulated p53 and down-regulated p21<sup>Cip1</sup> into S phase is an important trigger for apoptosis induced by transcriptional blockade. Several lines of evidence suggest that transcriptional blockade elicits cell death as cells enter S phase. 1) Synchronized cells released from nocodazole block in the presence of  $\alpha$ -amanitin underwent pronounced apoptosis during S phase (Fig. 5E). 2) Induction of G<sub>1</sub> arrest by treatment of cells with mimosine or by overexpression of p21<sup>Cip1</sup> or p27<sup>Kip1</sup> resulted in marked suppression of  $\alpha$ -amanitin-induced apoptosis (Fig. 6D). 3) Withdrawal of mimosine from  $\alpha$ -amanitin-treated cells led to massive apoptosis (Fig. 6C). Inhibition of CDK activity and induction of cell cycle arrest by p21<sup>Cip1</sup> has been shown previously to play an important role in protecting cells from apoptosis (51, 52). Together, these various observations suggest that transcriptional block by inhibition of pol II activity results in cell cycle arrest during DNA replication and induces apoptosis in S phase.

*Transcriptional Blockade Induces Translocation of p53 to Mitochondria and Consequent Apoptosis in S Phase*—Many genes whose products mediate p53-dependent cell death have been identified. Several p53 target genes encode proteins that localize to mitochondria and affect the mitochondrial membrane potential, an important determinant of mitochondrial apoptotic signaling. These proteins include Bax, Noxa, PUMA, and p53AIP1. We found that the accumulation of p53 induced by  $\alpha$ -amanitin did not result in the transcriptional activation of any of the p53 target genes examined. Furthermore, the abundance of Bax and Apaf-1, which are also p53-inducible gene products (53), was not affected by  $\alpha$ -amanitin treatment in HCT116 cells (supplemental Fig. S11). These observations thus suggest that apoptosis induced by transcriptional blockade is mediated by p53 in a transcription-independent manner.

Evidence suggests that p53 is able to directly engage the major apoptotic pathways in the cell, promoting both death receptor signaling and mitochondrial perturbations, without requiring gene induction. A fraction of accumulated p53 molecules also translocates to the mitochondria and thereby promotes apoptosis in cells with DNA damage (15). The p53 protein directly induces permeabilization of the outer mitochondrial membrane by forming complexes with the protective proteins Bcl-x<sub>L</sub> and Bcl-2, resulting in the release of cytochrome *c* into the cytosol. It has also been shown to activate and to induce the translocation of Bax to mitochondria, again resulting in cytochrome *c* release (54). However, specific cellular contexts that trigger the transcription-independent translocation of p53 to mitochondria remain obscure. We found that

$\alpha$ -amanitin-mediated transcriptional blockade induces p53 localization to mitochondria and that a substantial proportion of Bax molecules also translocates to mitochondria in  $\alpha$ -amanitin-treated MRC-5 cells (supplemental Fig. S12). Moreover, the p53 molecules that translocated to mitochondria in  $\alpha$ -amanitin-treated cells included those phosphorylated on Ser<sup>15</sup> and Ser<sup>46</sup>, and such translocation was detected in cells at S phase. Based on this evidence, we speculate that the p53 molecules that accumulate in response to transcriptional blockade trigger apoptosis directly by acting at mitochondria.

*Biological Significance of p53-mediated Apoptosis Induced by Transcriptional Blockade*—The induction of DNA lesions by high doses of UV radiation results in the ubiquitination and degradation of pol II LS, transcriptional collapse, and recruitment of DNA repair proteins (16, 17). Prolonged inhibition of transcription as a result of a failure to repair such DNA lesions results in cell death by apoptosis (18). Indeed, we found that high dose UV radiation induced pol II degradation, p53 accumulation, down-regulation of p21<sup>Cip1</sup> expression, and p53-dependent apoptosis, similar to the effects of  $\alpha$ -amanitin treatment. UV irradiation was shown previously to induce transcription of the *c-jun* gene and to modulate the association of p53 with the promoter of the p21<sup>Cip1</sup> gene, resulting in repression of p21<sup>Cip1</sup> gene activation and cell death (55). Therefore, in addition to the c-Jun-mediated pathway, signaling triggered by transcriptional blockade at the sites of unrepaired DNA lesions may contribute to UV-induced apoptosis by a p53-dependent pathway.

It is interesting to know how much the transactivation-independent apoptosis contributes to the p53-mediated cell death. When HCT116 (p53<sup>+/+</sup>) cells were treated with adriamycin in the presence of  $\alpha$ -amanitin, the cell death was significantly enhanced in comparison to adriamycin treatment alone even though the p53-mediated gene expression is blocked (supplemental Fig. S13). This observation implies that p53 accumulated by DNA damage can elicit apoptosis via both transactivation-dependent and -independent pathways.

It has been reported recently that many of the common DNA-damaging agents such as cisplatin and the topoisomerase I inhibitor camptothecin are known to interfere with transcription (56). Therefore, translocation of p53 to mitochondria may serve as a fail-safe mechanism to elicit apoptosis for eliminating cells whose transcription is blocked by severe and irreparable DNA damages (supplemental Fig. S14). Loss-of-function mutations in the p53 gene might thus abrogate both the transcription-dependent and mitochondrion-dependent apoptotic activities of p53 and thereby lead to the survival of transformed cells.

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