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p53-Dependent p21 mRNA Elongation Is Impaired when DNA Replication Is Stalled▽†

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We have previously reported that when DNA replication is blocked in some human cell lines, p53 is impaired in its ability to induce a subset of its key target genes, including *p21*$^{WAF1/CIP1}$. Here, we investigated the reason for this impairment by comparing the effects of two agents, hydroxyurea (HU), which arrests cells in early S phase and impairs induction of p21, and daunorubicin, which causes a G_2 block and leads to robust activation of p21 by p53. HU treatment was shown to inhibit p21 mRNA transcription rather than alter its mRNA stability. Nevertheless, chromatin immunoprecipitation assays revealed that HU impacts neither p53 binding nor acetylation of histones H3 and H4 within the *p21* promoter. Furthermore, recruitment of the TFIID/TATA-binding protein complex and the large subunit of RNA polymerase II (RNA Pol II) are equivalent after HU and daunorubicin treatments. Relative to daunorubicin treatment, however, transcription elongation of the *p21* gene is significantly impaired in cells treated with HU, as evidenced by reduced occupancy of RNA Pol II at regions downstream of the start site. Likewise, in the *p21* downstream region after administration of HU, there is less of a specifically phosphorylated form of RNA Pol II (Pol II–C-terminal domain serine 2P) which occurs only when the polymerase is elongating RNA. We propose that while the DNA replication checkpoint is unlikely to regulate the assembly of a *p21* promoter initiation complex, it signals to one or more factors involved in the process of transcriptional elongation.

The p53 tumor suppressor protein is a sequence-specific transcription factor that is found mutated in over 50% of human cancers (47, 70). p53 serves as a molecular guardian that responds to various forms of stress by regulating the expression of numerous genes involved in cell cycle progression, senescence, apoptosis, and mitosis (57, 58, 71). The ability of p53 to serve as a transcriptional regulator is essential to its roles in tumor suppression. The vast majority of tumor-derived mutations of p53 occur within the core DNA-binding domain of the protein, and such mutations usually prevent p53 from binding to promoters and activating target genes (33). Loss of p53 activity can result in uncontrolled cell proliferation in the presence of DNA damage, accumulation of mutations, and neoplasia (63). To date, hundreds of target genes have been identified for p53 (48, 71, 73), with the most well characterized of these promoting either cell cycle arrest or apoptosis. These genes include a G_1 cyclin-dependent kinase inhibitor, *p21*$^{WAF1/CIP1}$ (hereafter referred to as *p21*) (21, 36); an inhibitor of the G_2 cyclin B/CDK1 complex, *GADD45* (39, 77); a p53 E3-ubiquitin ligase, *MDM2* (4, 75), and proapoptotic genes, such as *PUMA*, *BAX*, *PIG3*, *p53AIP1*, *NOXA*, and *APAF1*, to name a few (37, 56, 72). There is also a significant body of evidence implicating cytoplasmic p53 in a transcription-independent mitochondrial apoptosis pathway (12, 16, 52), although recent work indicates interplay between the nuclear transcriptional activity of p53 and its functions in the cytoplasm (11).

It is well established that in response to DNA damage caused by ionizing radiation (IR), UV light, and a plethora of other agents (including topoisomerase inhibitors, such as daunorubicin [dauno]), the half-life ($t_{1/2}$) of p53 increases and cells undergo arrest in G_1 and/or G_2 (25). Following DNA damage, p53 becomes phosphorylated, acetylated, and subjected to other modifications which affect its stability, activity, and subcellular localization (2, 9, 59). Although most reports to date have studied the role genotoxic stress plays in activating p53, it has become evident that other forms of stress may also lead to the activation of the p53 response (57). In this study, we examined DNA replication block and the effect it has on p53 function.

Hydroxyurea (HU) is a chemotherapeutic drug that has been used in the treatment of leukemia (26). HU inhibits the function of ribonucleotide reductase, an enzyme responsible for the conversion of ribonucleotides (rNTPs) to deoxyribonucleotides (dNTPs) (65, 69). Upon treatment with HU, the cell's limited supply of dNTPs rapidly decreases, leading to stalled replication forks and a reversible, p53-independent arrest in early S phase. We previously showed that in response to the DNA replication block induced by HU, p53 protein is stabilized but is impaired in its ability to activate several but not all of its transcriptional target genes (32). Most relevantly, p21 protein and RNA fail to accumulate, while another p53 target, PIG3, exhibits no such accumulation defect. Since the expression of only a subset of p53 target genes is affected by DNA replication block, this impairment does not result from a global defect in transcription or translation after drug treatment, but instead points to a bona fide gene-selective mechanism. In a subsequent publication, we showed that reduction of p21 protein levels during S-phase arrest is critical for the cell to

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efficiently resume the cell cycle once the replication block is resolved (30). We further found that, after replication block, p21 protein is subjected to enhanced proteasome-dependent degradation. Thus, at least in some cell lines, the failure to accumulate p21 results from two different mechanisms, transcriptional impairment and increased proteolysis. Here, we investigated the reasons for the impairment in p21 mRNA accumulation following DNA replication block.

MATERIALS AND METHODS

Cell culture, FACS analysis, and drugs. Parental RKO and HCT116 cells (colorectal carcinoma cells expressing wild-type p53) and H1299 cells (p53-null lung epithelial carcinoma cells) were grown in Dulbecco modified Eagle medium containing 10% fetal bovine serum at 37°C. RKO-E6 (clone 10.2) cells, which stably express the human papillomavirus 16 (HPV-16) E6 protein, or RKO-neo cells, which have been stably transfected with an empty vector, were grown in McCoy's medium containing 10% fetal bovine serum supplemented with 500 μg/ml G418 and maintained at 37°C. These cells lines were gifts from K. Cho (29, 40). Drugs used in this study are as follows: hydroxyurea, 1.7 mM (Sigma); daunorubicin, 0.22 μM (Sigma); actinomycin D, 0.4 μM (Calbiochem). Flores-cence-activated cell sorter (FACS) analysis was performed as previously described (32).

Protein extraction and immunoblotting. Cells were lysed using HEPES lysis buffer (10 mM HEPES, pH 7.9; 1 mM EDTA; 10% glycerol; 400 mM NaCl; 1% NP-40; 0.5 mM phenylmethylsulfonyl fluoride, and protease inhibitors [1 μM benzamidine, 3 μg/ml leupeptin, 0.1 μg/ml bacitracin, 1 μg/ml macroglobulin]). Lysates were centrifuged to remove cellular debris, and whole-cell extract (40 μg) was resolved by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Membranes were probed using the following antibodies: p53, 1801 (supernatant solution from hybridoma culture); p21, Waf-1 (Ab-1) (OP-64; Oncogene Research); actin, A-2066 (Sigma). Anti-mouse or anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Sigma) were incubated with membranes and detected by enhanced chemiluminescence (Amersham).

RNA extraction and RT-PCR analysis. Cells were seeded and treated according to the conditions described in the figure legends for each experiment. RNA was harvested using a QIAGEN RNeasy Mini kit according to the manufacturer's specifications. First-strand cDNA synthesis and reverse transcriptase (RT)-PCRs were performed as previously described (51). Initial RT-PCRs were performed in parallel with a standard curve of template DNA, and different cycling conditions were tested to confirm that amplifications were within the linear range. A "No RT" reaction, in which RNA was subjected to the conditions of cDNA synthesis without reverse transcriptase, was included as a negative control in RT-PCR experiments to confirm the purity of RNA samples. In most cases, RT-PCRs were run using 2.5% agarose gels, imaged with a Gel Logic 100 imaging system (Kodak), and quantitated with Kodak 1-D image analysis software. For PCRs conducted in the presence of radioactive dCTP, samples were resolved on 8% polyacrylamide gels, dried, and autoradiographed. In these instances, band quantitation and analysis was performed using a phosphorimager (Molecular Dynamics). Primer construction is as follows: RT-p21 forward (Fwd), 5′-GTTCCTTGTGGAGCCGGAGC-3′; RT-p21 reverse (Rev), 5′-GGTACAA GACAGTGACAGGTC-3′; RT-PIG3 Fwd, 5′-GCAGCTGCTGGATTCAATT AC-3′; RT-PIG3 Rev, 5′-GCCTATGTT CTTGTTGGCCTC-3′; RT-MDM2 Fwd, 5′-GTGAATCTACAGGGACGCCAT-3′; RT-MDM2 Rev, 5′-CTGATC CAACCAATCACCTGAA-3′; RT-PUMA Fwd, 5′-TGTGAATCCTGTGCTCT GCC-3′; RT-PUMA Rev, 5′-TTCCGGTATCTACAGCAGCG-3′; RT-GAPDH Fwd, 5′-CAACTACATGGTTTACATGTTC-3′; RT-GAPDH Rev, 5′-GCCAG TGGACTCCACGAC-3′.

RNA stability experiments. First, RKO cells were treated with either hydroxyurea (1.7 mM, 24 h) or daunorubicin (0.22 μM, 12 h) to stabilize p53 protein to an equivalent degree in the two experimental sets. Next, 0.4 μM actinomycin D was added to the culture media and incubated for 0, 1, 2, 4, or 6 h. Cells were washed twice with phosphate-buffered saline, harvested by scraping, and divided into two samples. Cells in each aliquot were pelleted by centrifugation and subjected to either RNA extraction followed by RT-PCR or protein extraction followed by immunoblotting as described above.

Slot blots and nuclear run-on assays. Slot blots were generated as follows. Plasmid DNA containing cDNAs of interest was linearized and purified by phenol-chloroform/isoamyl alcohol extraction followed by ethanol precipitation. Linearized plasmids were boiled in the presence of 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and single-stranded DNA was slot blotted on Nytran-N membrane (Schleicher and Schuell Bioscience, Inc.) that was previously wetted in 2× SSC. Blots were UV cross-linked (autolink setting, UV Crosslinker; Fisher Biotech) and incubated overnight in 1 ml of prehybridization buffer (2× TESS [20 mM Tris {pH 7.4}, 10 mM EDTA, 0.3 M NaCl, 0.2% SDS], 4× Denhardt's reagent, 100 μg/ml tRNA) at 65°C (with rocking).

To isolate nuclei, approximately 5×10^6 cells were washed in cold phosphate-buffered saline, scraped, and pelleted by centrifugation ($300 \times g$ for 5 min). The cells were resuspended in 2 ml of NP-40 lysis buffer (10 mM Tris, pH 7.4; 10 mM NaCl; 3 mM $MgCl_2$; 0.5% NP-40) and centrifuged ($1{,}175 \times g$ for 5 min) to pellet nuclei, and the pellet was washed with 4 ml NP-40 lysis buffer. Nuclei were centrifuged once again, and the pellet was resuspended in 100 μl nuclear storage buffer (50 mM Tris, pH 8.3; 40% [vol/vol] glycerol; 5 mM $MgCl_2$; 0.1 mM EDTA) and stored at −70°C.

Run-on reactions were performed as follows. Nuclei were thawed on ice, mixed with 100 μl reaction buffer (10 mM Tris, pH 7.5; 10 mM $MgCl_2$; 300 mM KCl; 0.5 mM each of ATP, CTP, and GTP; 15 μl [α-^{32}P]UTP [3,000 mCi/ml]), and incubated at 30°C for 30 min. The reactions were stopped by the addition of 0.5 ml TRIzol reagent (Invitrogen), and labeled RNA was extracted according to the manufacturer's specifications. RNA was resuspended in RNase-free water and added to the hybridization solution (2× TESS; 1× Denhardt's reagent; 100 μg/ml tRNA). Approximately 6×10^6 cpm of probe was used for each sample. Blots were hybridized for 48 h at 65°C (with rocking) and then washed twice in 2× SSC/0.2% SDS, washed twice in 0.2× SSC/0.2% SDS (20 min per wash at 65°C), dried briefly, and exposed. Bands were quantitated and analyzed by phosphorimaging.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) assays were performed as previously described (51). The antibodies used for ChIP were p53, PAb1801/DO-1 cocktail (supernatant solution from hybridoma cultures); acetyl-histone H3, 06-599 (Upstate Biotechnology); acetyl-histone H4, 06-598 (Upstate Biotechnology); Pol II (N-20), sc-899 (Santa Cruz); Pol II-CTD serine 2P (H5), MMS-129R (Covance); and TFIID/TBP (N-12), sc-204 (Santa Cruz). In cases where immunoprecipitations were performed using antibodies directed against acetylated histones, 5 mM sodium butyrate and 5 μM trichostatin A were also added to the lysis buffer to protect against histone deacetylase activity. In cases where immunoprecipitations were performed using antibodies against the phosphorylated form of RNA Pol II (anti-Pol II–C-terminal domain [CTD] serine 2P), a mixture of phosphatase inhibitors (Calbiochem) was added to the lysis buffer to protect against phosphatase activity, and the beads/antibody used for immunoprecipitation were prepared as previously described (43). PCRs were carried out for 30 cycles unless otherwise indicated. Linear amplification of PCRs was confirmed, and amplicons were resolved by the same methods as those described for RT-PCR. ChIP primer sequences are as follows: p21 promoter (5′ p53 response element) (28 cycles) Fwd, 5′-CTGGACTGGGCACTCTTGTC-3′, and p21 promoter Rev, 5′-CTCCTACCATCCCCTTCCTC-3′; p21 TATA promoter Fwd, 5′-TATTGTGGGGCTTTTCTG-3′, and p21 TATA promoter Rev, 5′-CTGTTAGAATGAGCCCCCTTT-3′; p21 NEG promoter Fwd, 5′-GGAGT CCTGTTTGCTTCTGG-3′, and p21 NEG promoter Rev, 5′-CTTTGGCCACA CTGAGGAAT-3′, and p21 Intron 1 Fwd, 5′-GTGATGGGCCTCTCTGGTTA-3′; p21 Intron 1 Rev, 5′-TGTTTGGGGGCTGTTCTAAG-3′; p21 Exon 2 (28 cycles) Fwd, 5′-GAGCGATGGAACTTCGACTT-3′, and p21 Exon 2 Rev, 5′-CAGGTCCACATGGTCTTCCT-3′; p21 Exon 3 (28 cycles) Fwd, 5′-CCCTTT CCTGGACACTCAGA-3′, and p21 Exon 3 Rev, 5′-CCCTAGGCTGTGCTCA CTTC-3′; MDM2 promoter Fwd, 5′-TCGGGTCACTAGTGTGAACG-3′, and MDM2 promoter Rev, 5′-CACTGAACACAGCTGGGAAA-3′; GADD45 promoter Fwd, 5′-GGTTGCCTGATTGTGGATCT-3′, and GADD45 promoter Rev, 5′-GAGTAGCTGGGCTGACTGCT-3′; PIG3 promoter Fwd, 5′-CACTC CCAACGGCTCCTTT-3′, and PIG3 promoter Rev, 5′-GCCCATCTTGAGCA TGGGT-3′; PIG3 Exon 2 Fwd, 5′-CCAGCAACATTTTGGGACTT-3′, and PIG3 Exon 2 Rev, 5′-TCTGGGATAGGCATGAGGAG; and PIG3 Exon 3 Fwd, 5′-ATGCAGGACTGAGTGGTGTG-3′, and PIG3 Exon 3 Rev, 5′-GCGTTGCT TCAGAGAAATCC-3′; GAPDH promoter Fwd, 5′-AAAAGCGGGGAGAAAG TAGG-3′, and GAPDH promoter Rev, 5′-CTAGCCTCCCGGGTTTCTCT-3′.

RESULTS

p53 is transcriptionally impaired in response to DNA replication block. For the present study, we compared the effects of HU, which causes cells to accumulate at the G_1/S-phase border with those caused by dauno, a topoisomerase II inhibitor (45) that arrests RKO colorectal cancer cells in G_2 (see

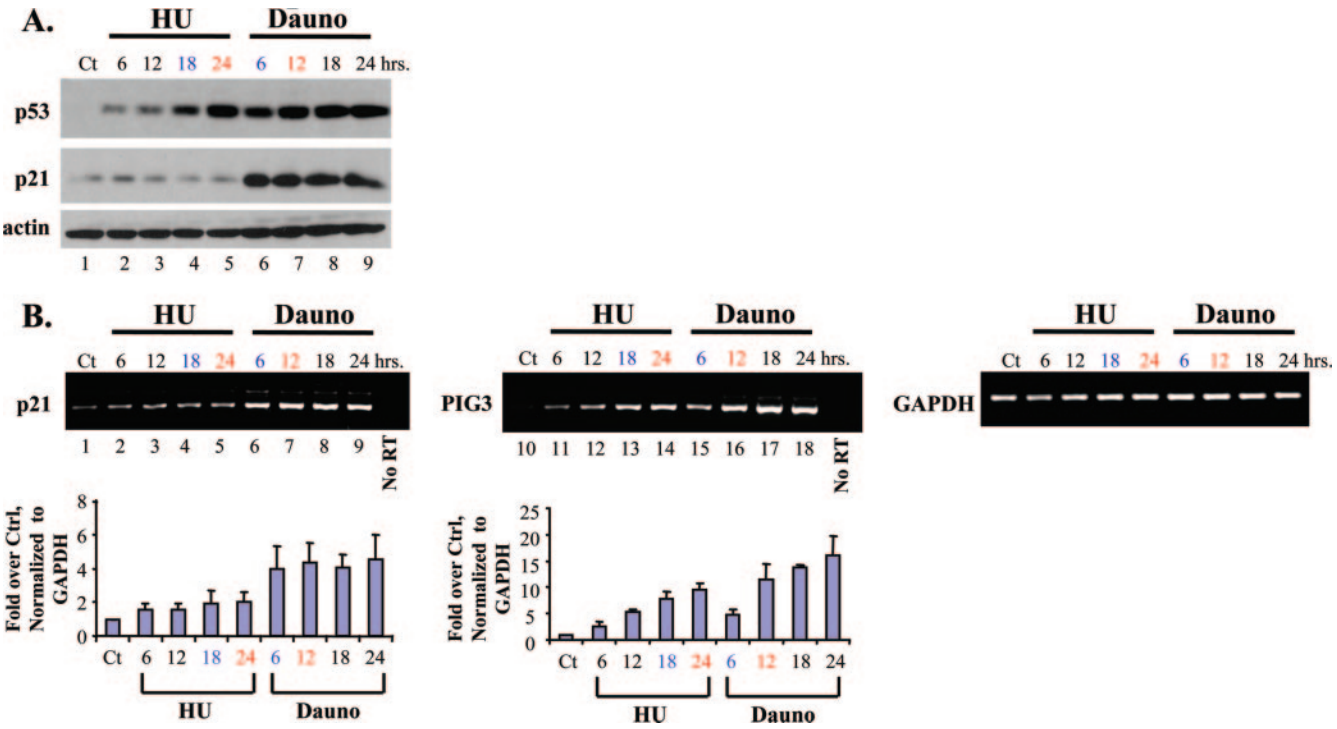

FIG. 1. HU treatment selectively impairs activation of p53 target genes. (A) RKO cells were untreated (Ct) or treated with HU (1.7 mM), or daunorubicin (0.22 μM) over a time course (6 to 24 h, as indicated), lysed, and subjected to immunoblot analysis with anti-p53, -p21, and -actin antibodies. Red- and blue-highlighted time points indicate respective conditions in which equivalent levels of p53 protein have been induced. (B) RKO cells were treated as for panel A, and RNA was extracted and subjected to RT-PCR using primer sets for the indicated genes. Amplicons were resolved by agarose gel electrophoresis and stained using ethidium bromide. Graphs show the averages from three independent experiments, and error bars included in the graphs represent one standard deviation. Ctrl, control.

Fig. 2D and E and reference 32). The first objective was to find circumstances where HU or dauno treatment could induce equivalent amounts of p53 protein and determine the cell's response to these conditions. RKO cells were treated with HU or dauno for various times, and then p53 and p21 protein levels were assessed by immunoblotting (Fig. 1A). We also examined mRNA levels of both p21 and another p53 target, PIG3, under these conditions (Fig. 1B). Time points were identified where equivalent levels of p53 protein were detected (Fig. 1A, top panel, compare lanes 4 and 6 or lanes 5 and 7). At these times, far less p21 protein accumulated in the samples treated with HU than in the related samples treated with dauno (Fig. 1A, middle panel). The inefficient accumulation of p21 after HU treatment was linked to impaired transcription of the *p21* gene as measured by RT-PCR, which was observed very early and was sustained throughout the time course (Fig. 1B, left panel and graph, compare lanes 2 to 5 with lanes 6 to 9). In contrast, even very early (6 h) after dauno treatment, there was strong activation of the *p21* gene, indicating that submaximal levels of p53 were capable of fully transactivating the *p21* promoter. On the other hand, under conditions where p53 levels were equivalent, the *PIG3* promoter was transactivated to similar extents with the two treatments (Fig. 1A; Fig. 1B, middle panel). In fact, in the HU 12-h sample, PIG3 mRNA accumulated to an extent similar to that seen 6 h after dauno, despite the fact that the p53 protein level in the HU sample was approximately half of that seen for dauno (Fig. 1A, compare lanes 3 and 6; Fig. 1B, compare lanes 12 and 15). These results demonstrate the selective impairment of p53 transcriptional readout following HU treatment in RKO cells.

RKO cells were also treated with a combination of HU and dauno to assess the impact both drug treatments together would have on p53 accumulation and its downstream targets (Fig. 2). Under conditions where p53 levels were similar and dauno treatment alone provoked a robust accumulation of p21 protein and mRNA, the cotreatment of cells with HU and dauno led to extremely low p21 protein levels that were similar to those with HU alone (Fig. 2A). Similarly, with a combination of HU and dauno treatment, p21 mRNA was induced less well than with dauno alone, although it accumulated slightly better than with HU alone (Fig. 2B, compare lanes 3 and 4). As before, the p53 target gene *PIG3* yielded similar levels of accumulated mRNA in response to both HU and dauno treatments, with somewhat more PIG3 mRNA being detected upon cotreatment with these drugs. Interestingly, this response might be common among proapoptotic targets of p53, as a RT-PCR analysis of the *PUMA* gene shows results similar to those for *PIG3* (Fig. 2B, compare lanes 12 to 14 to lane 11).

In line with these results, the cell cycle distribution caused by HU treatment predominated when cells were cotreated with either gamma irradiation or daunorubicin (Fig. 2C and D). When cells were blocked in S phase by aphidicolin (an inhibitor of DNA polymerases), the results were essentially similar, indicating that the effects of HU are not due to a specific effect of this drug on cells but rather reflect its function in blocking S phase (Fig. 2E; see Fig. S1A and B in the supplemental

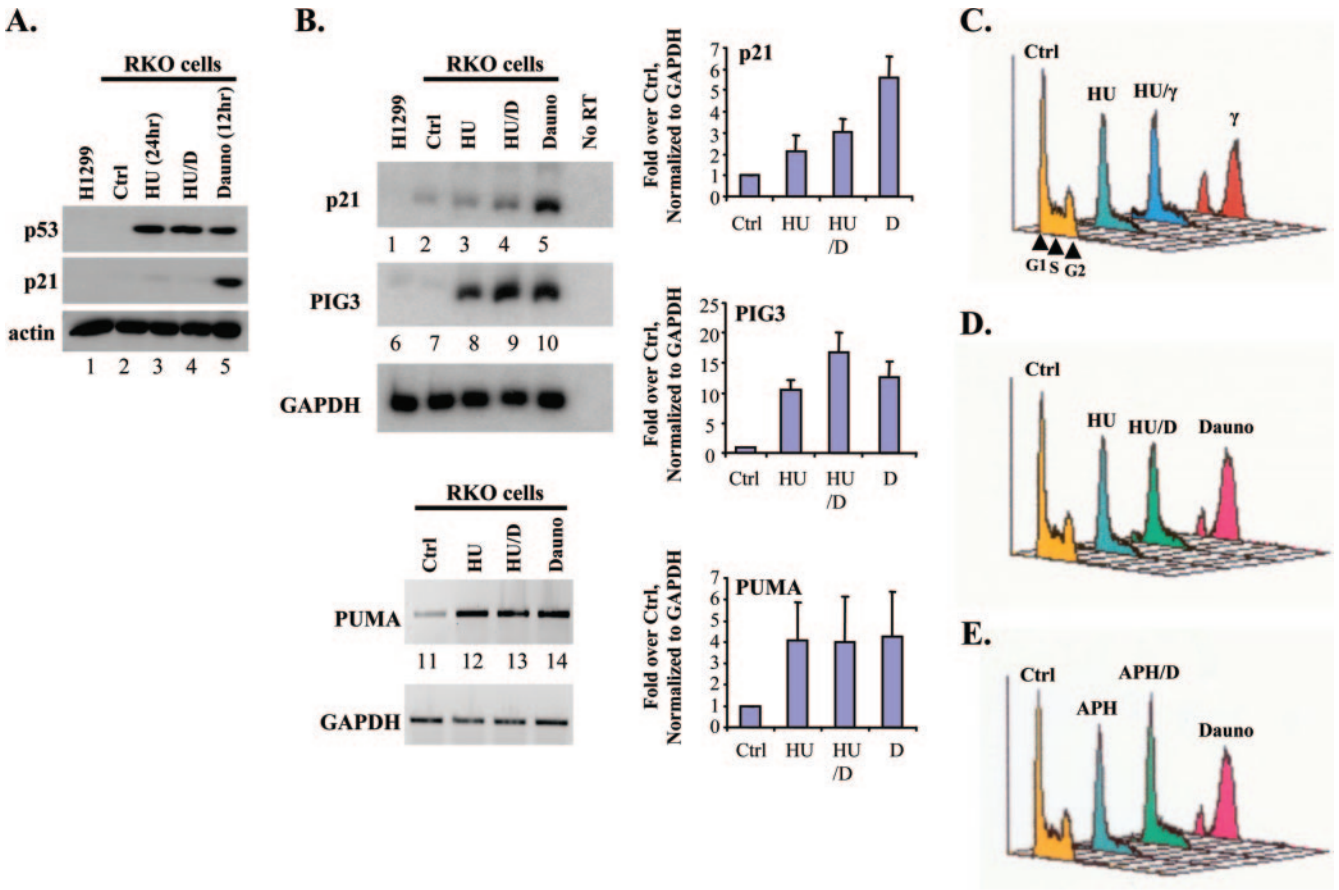

FIG. 2. Stalled DNA replication represses the p53 response to daunorubicin. (A) RKO cells were untreated (Ctrl) or were treated with HU and dauno (as described for Fig. 1A) for 24 and 12 h, respectively, as indicated. For the "HU/D" sample, cells were pretreated with HU for 12 h and then daunorubicin was added to the culture medium, which was incubated in combination with HU for an additional 12 h. H1299 cells were untreated and included as a negative control. Cells were collected, lysed, and subjected to immunoblot analysis using the indicated antibodies. (B) H1299 and RKO cells were treated (as described for panel A), and RNA was extracted and subjected to RT-PCR analysis. [α-^{32}P]dCTP was included in the PCR mixture for *p21*, *PIG3*, and *GAPDH* (top) to obtain radioactive amplicons. For *PUMA* RT-PCR experiments (and the accompanying GAPDH [bottom] experiments), PCRs were conducted in the absence of [α-^{32}P]dCTP, and amplicons were resolved by agarose gel electrophoresis. Graphs show the averages and standard deviations (error bars) for two independent RT-PCR experiments. (C to E) FACS analysis was performed using RKO cells that were subjected to different combinations of drug treatments as shown, and data were analyzed using the ModFit program. (C) Cells were treated with HU (24 h), with gamma IR (10 Gy) for 12 h (γ), or a combination of both treatments (HU for 12 h and then gamma IR for an additional 12 h [HU/γ]). (D) RKO cells were treated as described for panel A. (E) RKO cells were treated with aphidicolin (APH, 5 μg/ml) for 24 h, dauno for 12 h, or a combination of both treatments (APH for 12 h then dauno for an additional 12 h [APH/D]). Ctrl, control; D, dauno.

material). Note as well that the HU-related impairment of p53 transcriptional activity was also seen in another cell line, HCT116 (see Fig. S1C and D in the supplemental material). Thus, when DNA replication is stalled, cells cannot respond to a typical p53 stress signal.

Although the transcriptional activity of p53 was drastically impaired following DNA replication block, it was not completely eliminated (Fig. 1B, compare levels of p21 mRNA in control and HU-treated samples). To determine if this small amount of p21 accumulation was dependent on p53, we compared the effects of HU in a cell line (RKO-E6) engineered to express a stable form of the HPV-16 E6 protein with a control cell line (RKO-neo) (29, 40). Since the HPV-16 E6 protein targets p53 for degradation by the proteasome (60), RKO-E6 cells have extremely low levels of p53 protein and are incapable of stabilizing p53 following stress. In fact, the results showed that after HU treatment of RKO cells, the small amount of p21 mRNA that accumulates is independent of p53 (see Fig. S2 in the supplemental material). Therefore, p53-mediated induction of p21 is specifically targeted by stalled DNA replication.

The rate of transcription from the *p21* promoter is decreased in HU-treated cells. Although HU treatment impaired the ability of p53 to induce accumulation of p21 mRNA, it was unclear whether this resulted from inhibition of p21 mRNA transcription or decreased mRNA stability. To address this issue, we used actinomycin D to halt new transcription and monitor the degradation rates of p21 mRNA over time (50, 66). First, RKO cells were treated with HU for 24 h or dauno for 12 h to allow accumulation of equivalent levels of p53 protein and were then treated with actinomycin D. Cells were harvested at increasing time points, and samples were prepared for immunoblotting (Fig. 3A) or RT-PCR (Fig. 3B). While the half-life of p21 mRNA was slightly higher in drug-treated cells than in untreated cells (control $t_{1/2}$, 3 h; HU and

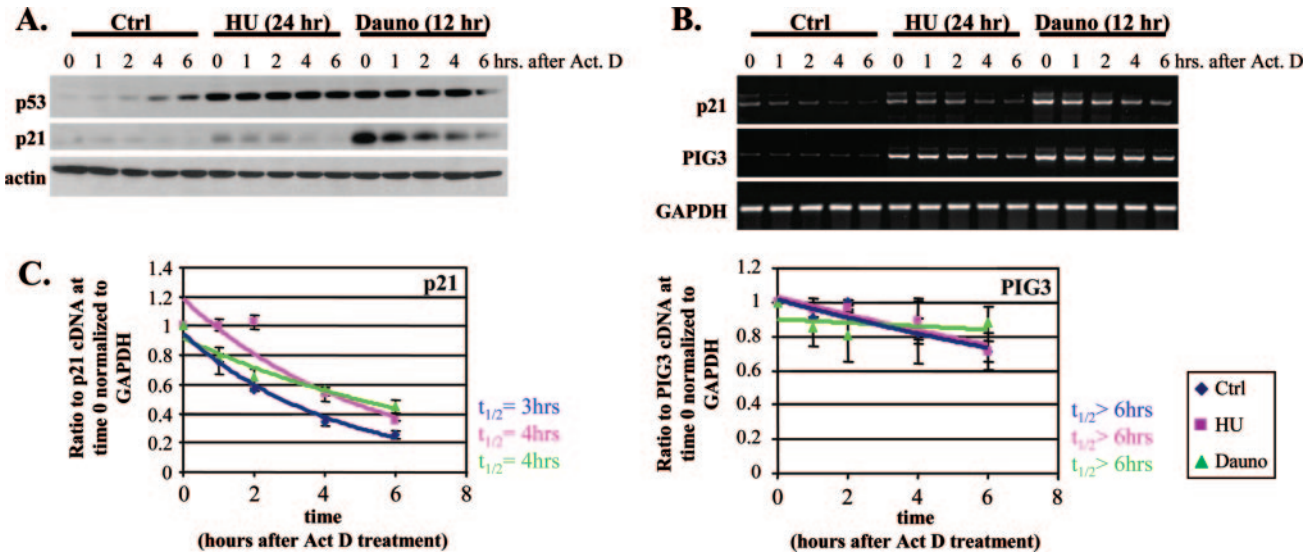

FIG. 3. mRNA stability of p53 target genes is not altered after HU treatment. (A and B) RKO cells were either untreated (Ctrl) or treated with HU for 24 h or daunorubicin for 12 h, followed by treatment with 0.4 μM actinomycin D (Act. D) for the time periods indicated. Cells were harvested, divided into two aliquots, lysed, and subjected to analysis by immunoblotting using the indicated antibodies (A) or RNA extraction and RT-PCR using primer sets for the indicated genes (B). (C) RT-PCR data from experiments shown in panel B was quantitated using Kodak 1-D image analysis software. Each point was plotted as a ratio of the amplified cDNA that exists at time zero. Half-life measurements were extrapolated from the graphs for each treatment used. Graphs show the averages for three independent experiments, with error bars representing one standard deviation.

dauno $t_{1/2}$, 4 h), there were no significant differences between HU and dauno-treated cells in the half-lives of p21 mRNA (Fig. 3C). Moreover, although PIG3 mRNA was extraordinarily stable, making it difficult to reliably estimate its half-life under these conditions ($t_{1/2}$ was >6 h in all cases), there was no change in PIG3 mRNA stability under each of these conditions. Likewise, another p53 target gene, *MDM2*, whose induction is impaired after HU treatment (32), exhibited no change in mRNA stability with these treatments (data not shown).

Steady-state RNA levels are the sum of RNA synthesis and RNA degradation. Since differences in mRNA degradation had been ruled out as the explanation for reduced p21 mRNA accumulation after HU treatment, we employed a nuclear run-on assay which can directly measure the rate of transcription of a particular gene in its native cellular context (20, 53). Nuclear run-on experiments performed in the presence of HU showed a clear decrease in the rate of transcription from the *p21* gene compared to daunorubicin (Fig. 4). Conversely, PIG3 mRNA synthesis showed no such decrease following HU treatment. Therefore, impaired p21 mRNA accumulation after HU treatment is the result of a defect in its synthesis rather than its posttranscriptional stability.

p53 binding to the p21 promoter is not affected by blocked DNA replication. An explanation for the decreased amount of

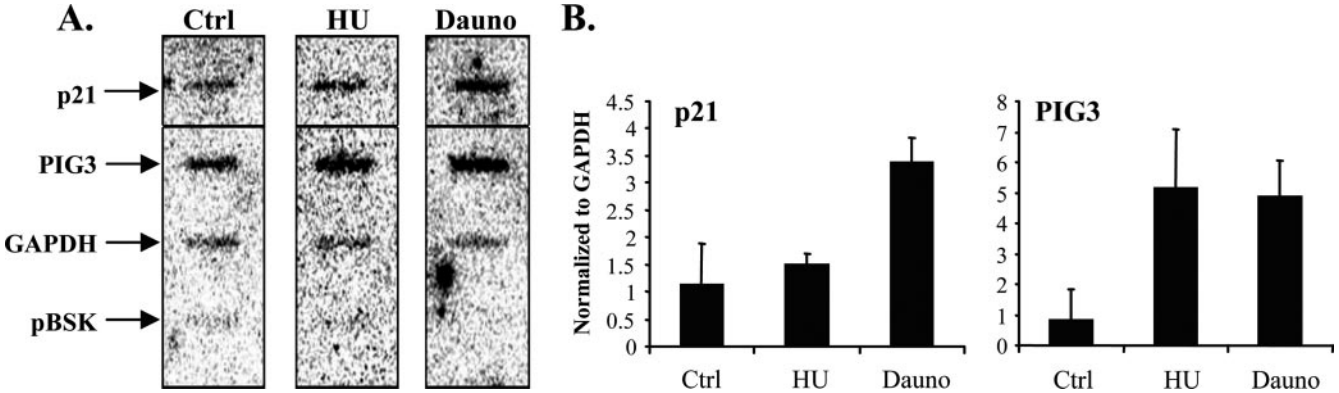

FIG. 4. HU treatment results in reduced transcription from the *p21* promoter. (A) Nuclear run-on assays were performed as described in Materials and Methods. Linearized plasmids containing cDNA fragments from the *p21*, *PIG3*, and *GAPDH* genes were immobilized on nylon membranes and hybridized with ^{32}P-labeled RNA probes generated from run-on transcription in RKO cells (control [Ctrl] or HU or dauno treated, as for Fig. 2A). Approximately 6×10^6 cpm of labeled total RNA was added to each hybridization reaction. Horizontal lines within the nuclear run-on images indicate that intervening slots have been spliced out. (B) Graphical representation of the averages and standard deviations (error bars) for three independent nuclear run-on experiments. Blots were analyzed by phosphorimaging, and background corrections were made for each blot by first subtracting the amount of hybridization to a negative control (linearized pBSK empty vector) and then normalizing data to the amount of GAPDH synthesis.

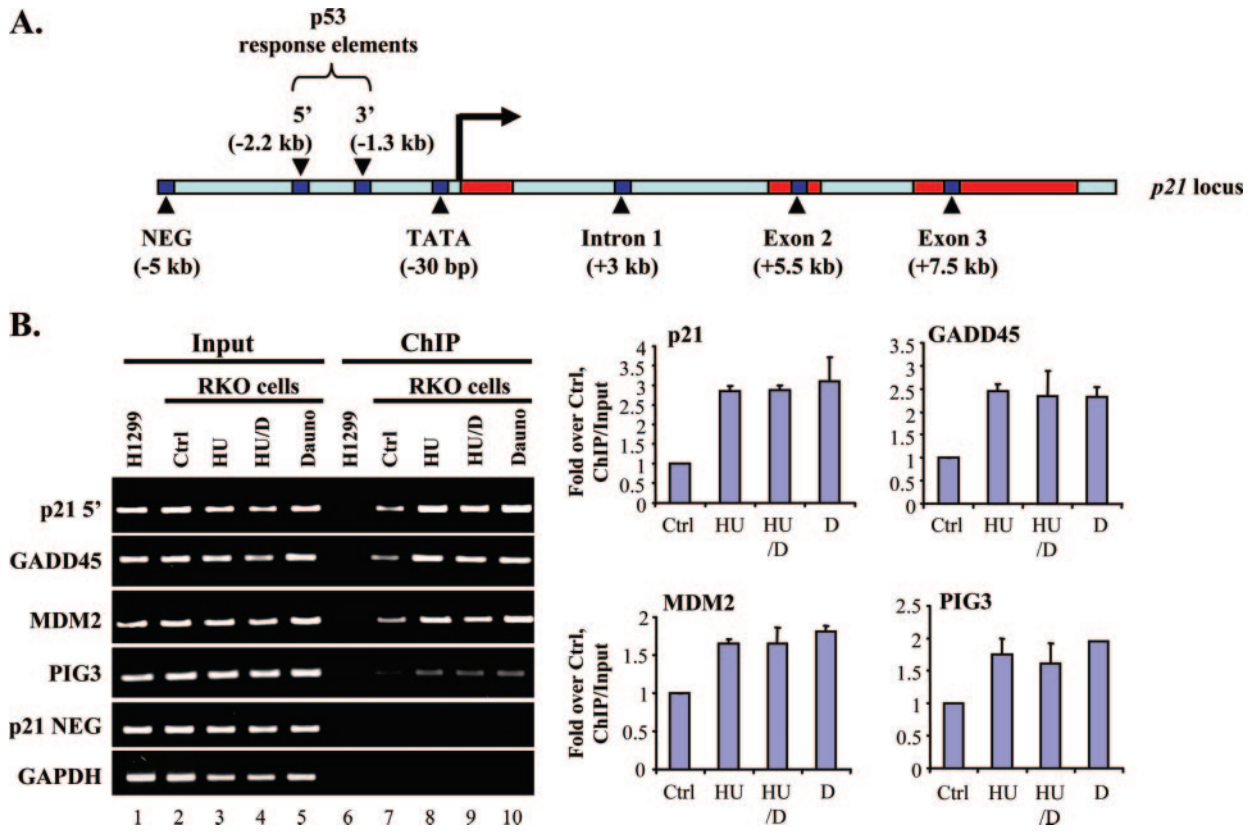

FIG. 5. p53 associates with *p21* and other target promoters in HU-treated cells. (A) Schematic representation of the *p21* locus. The locations of all PCR primers used in ChIP assays are labeled and indicated by dark blue boxes. Red areas indicate the approximate locations of exons within *p21*. The transcription start site (+1 bp) is indicated by a black, right-angle arrow. (B) p53 ChIP was performed in H1299 or RKO cells following HU and/or daunorubicin treatment. Cells were treated as described for Fig. 2A, and lysates were immunoprecipitated with anti-p53 monoclonal antibodies (1801 and DO-1). Approximately 0.2% of input chromatin and 0.5% of ChIP DNA was used as a template in the PCRs. Graphs show the averages and standard deviations (error bars) for two independent ChIP experiments and are representative of a total of five experiments. Data are expressed graphically as induction (*n*-fold) compared to untreated RKO cells. p21 NEG (2.8 kb upstream of the 5′ response element) and GAPDH primer sets were included as negative controls to confirm the specificity of the ChIP assay. Ctrl, control; D, dauno; HU/D, HU and dauno.

p21 transcription observed after DNA replication block could be an inability of the p53 protein to bind to the *p21* promoter. We used the ChIP assay to examine this possibility. Various regions within the *p21* locus, as well as a number of other target genes (*PIG3*, *GADD45*, and *MDM2*), were examined to detect bound p53 after treatments with HU, dauno, or a combination of both drugs (Fig. 5). Consistent with increased p53 levels caused by these treatments (Fig. 2A), there was in each case more p53 bound to these promoters than in untreated cells. With some genes, however (e.g., *MDM2* and *PIG3*), the increase in binding was not proportional to the increase in detectable p53 protein in treated versus untreated cells. The analyses revealed no apparent differences in the abilities of p53 to bind the 5′ response element within the p21 promoter following HU versus dauno treatment. Furthermore, other target genes tested, including the *PIG3* gene, showed no differences in the abilities of p53 to bind response elements within their promoters after these treatments (Fig. 5B). Similar results were obtained for HCT116 cells treated with HU and dauno, as well as for RKO cells treated with aphidicolin and dauno (data not shown). Additionally, p53 association with the proximal (3′) p53 response element within the *p21* promoter was examined and found to give results similar to those seen with the 5′ site in RKO cells after these treatments (data not shown). Several controls were performed to support the validity of these results. First, in different p53 ChIP experiments, both polyclonal and monoclonal antibodies were used and, in all cases, greater than 90% immunodepletion of p53 was observed, as determined by immunoblotting analysis of the postimmunoprecipitate supernatants (data not shown). Second, as a control for immunoprecipitation specificity, ChIP assays were performed using the p53-null H1299 cell line (Fig. 5B, lane 6) as well as the RKO-E6 cell line (data not shown), and virtually no ChIP signal was detected. Finally, p53 binding to a far-upstream region of the p21 promoter (−5 kb) was undetectable (Fig. 5B, p21 NEG panel). Taken together, these data indicate that the impairment in p53 transcriptional activity following DNA replication block does not result from a defect in its ability to occupy response elements within target promoters.

Impaired transcription of the *p21* gene is not the result of defective acetylation of core promoter histones H3 and H4. Acetylation of critical lysines within histones (along with methylation of other lysines) causes remodeling of chromatin in the vicinity of the modification and, in turn, facilitates transcription of the relevant gene (19, 24). p53 is known to be function-

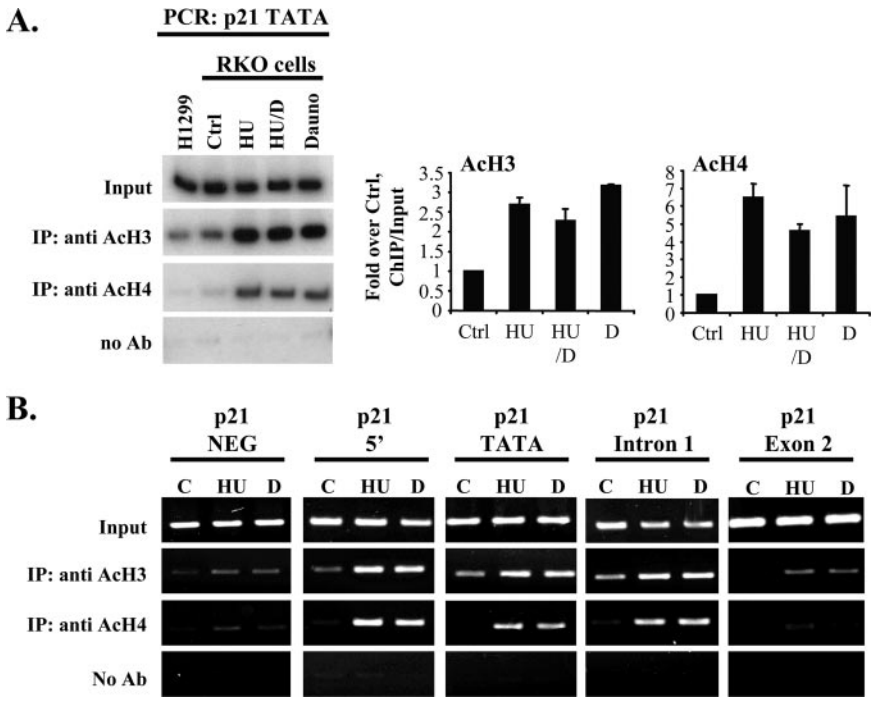

FIG. 6. HU treatment does not impair acetylation of histone H3 and H4 at the *p21* promoter. (A) H1299 and RKO cells were treated as described for Fig. 2A, and ChIP assays were performed to compare *p21* promoter histone acetylation following HU or daunorubicin treatment. Lysates were immunoprecipitated with polyclonal antibodies specific for acetylated histones H3 and histone H4. A sample in which lysates were incubated with beads lacking antibody (no Ab) was included to determine background levels of chromatin pulled down by beads alone. Approximately 0.2% of input chromatin and 0.5% of ChIP-DNA was used as a template in the PCRs with [α-^{32}P]dCTP in the reaction mix. Radioactive amplicons were resolved on 8% acrylamide gels, dried, and autoradiographed. ChIP data were analyzed by phosphorimaging and normalized to their respective inputs. Graphs show the averages and standard deviations (error bars) for two independent ChIP experiments, expressed as induction (*n*-fold) compared to untreated RKO cells. (B) Lysates from RKO cells were immunoprecipitated with acetylated histone antibodies (as described for panel A), and PCR was performed using primers to amplify different regions of the *p21* promoter and gene as indicated (see Fig. 5A for primer locations). Amplicons were resolved by agarose gel electrophoresis. Ctrl or C, control; D, dauno; HU/D, HU and dauno.

ally and physically associated with several acetyltransferases, including p300/CBP, PCAF, Tip60, and TRRAP (7, 15, 19). These interactions can lead to promoter histone acetylation and increased transcriptional activity (1, 3, 5, 22). We examined the status of histone acetylation within the *p21* promoter TATA region following HU and dauno treatment by performing ChIP assays with antibodies against the acetylated forms of histone H3 and H4 (Fig. 6). As a control for basal p53-independent histone acetylation within the *p21* promoter, we used H1299 cells, which are null for p53. Compared to untreated RKO cells, both HU and dauno treatments led to robust induction of acetylation of both histones, as did the combination of HU and dauno (Fig. 6A). Thus, although the overall cellular levels of acetyl-histone H3 and H4 protein were not increased following drug treatment (data not shown), the presence of these modified forms within the *p21* promoter was damage dependent and likely resulted from the binding of p53 protein to the promoter. There were no differences seen between HU or dauno treatments alone or together, indicating that nucleosomes at the *p21* promoter were modified to a similar extent whether they were being transcribed at low (HU) or high (dauno) levels.

When the extent of acetylated histones H3 and H4 was assessed within other regions of the *p21* gene (Fig. 5A, diagram of *p21* locus and primer positions), the highest level of acetylated histones occurred around the p53 5′ response element within the *p21* promoter, and strong signals were obtained as far downstream as intron 1 (+3 kb), correlating with both drug treatment and high levels of p53 binding (Fig. 6B). Conversely, very low levels of acetylated histones were detected both in a distal region of the *p21* promoter (p21 NEG, approximately 2.8 kb upstream of the 5′ p53 binding site, where no p53 binding is observed by ChIP) or within a region further downstream (p21 Exon 2, +5.5 kb). Even at these distal regions, somewhat more acetylation of these histones was still detected after drug treatment. Whether the extent of acetylation was relatively high or low, no differences between HU and dauno-treated cells were seen. Thus, although we cannot rule out subtle variations in acetylated histone H3 and H4 residues, our data indicate that HU-mediated transcriptional impairment occurs downstream of *p21* promoter histone acetylation.

Association of TATA-binding protein and RNA polymerase II (RNA Pol II) with the *p21* promoter is not affected by stalled DNA replication. Recruitment of the TATA-binding protein (TBP) as part of its holocomplex, TFIID, is one of the first events in the assembly of the transcription initiation machinery, and this protein has been shown to functionally and physically interact with p53 (10, 49, 61). We examined the ability of TBP to assemble at the *p21* promoter TATA box region following DNA replication block by using ChIP with anti-TBP

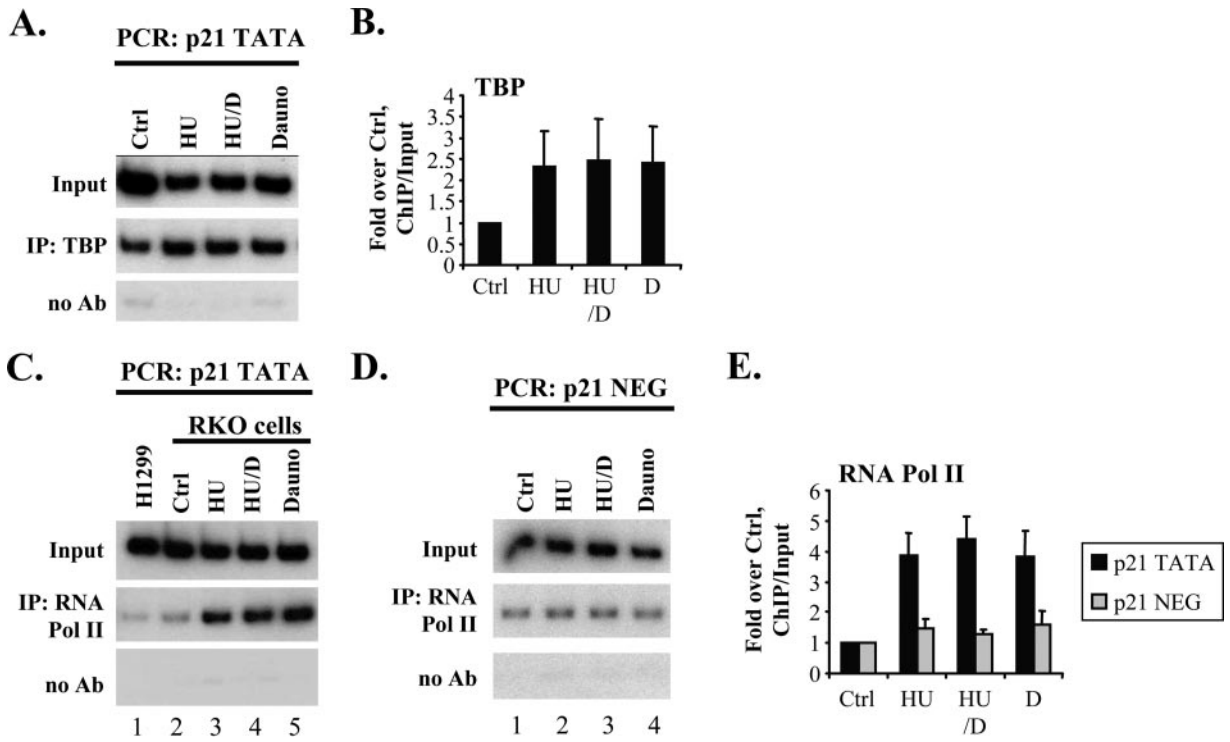

FIG. 7. TATA-binding protein and RNA polymerase II are recruited to the *p21* promoter following HU treatment. (A) RKO cells were treated as described for Fig. 2A, and ChIP assays were performed using lysates with a polyclonal TBP antibody to immunoprecipitate TBP/chromatin complexes. Approximately 0.2% of input chromatin and 4% of ChIP-DNA was used as a template in the PCRs including [α-^{32}P]dCTP in the reaction mix. (B) Graphical representation of the data shown in panel A. The graph shows the averages and standard deviations (error bars) for three independent experiments, expressed as induction (*n*-fold) compared to untreated RKO cells. (C to E) H1299 and RKO cells were treated as for Fig. 2A, and ChIP assays were performed using a polyclonal antibody against the large subunit of RNA Pol II. DNA isolated from anti-RNA Pol II ChIPs was subjected to PCR analysis (as in panel A) using primers for the *p21* TATA region (C, p21 TATA) or a *p21* 5′ distal region as shown in the diagram in Fig. 5A (D, p21 NEG). (E) Graphical representation of the data shown in panels C and D. Data are the averages from five (C, p21 TATA) or three (D, p21 NEG) independent experiments, with error bars representing one standard deviation. As in Fig. 6, ChIP assays were performed in parallel with a no-antibody control (no Ab) to determine background levels of chromatin pulled down by beads alone. Ctrl, control; D, dauno; HU/D, HU and dauno.

antibodies. Consistent with previous reports, basal binding by TBP was quite high (23, 27); however, an approximately 1.5- to 2.5-fold increase in occupancy was reproducibly seen upon damage. Here, too, there was no difference in TBP binding in cells treated with HU or daunorubicin (Fig. 7A and B).

Having found that bound p53, acetylated histones, and TBP recruitment were essentially unaffected by HU treatment, we went on to examine the recruitment of RNA Pol II to the *p21* promoter. ChIP assays were performed using antibodies against the largest subunit of RNA Pol II, and PCRs were performed using primers flanking the TATA box region (Fig. 7C). Recruitment of polymerase to the *p21* promoter TATA box region occurred in a stress- and p53-dependent manner (Fig. 7C, compare lane 1 and 2 to lanes 3 to 5; Fig. 7E), and the significant presence of RNA Pol II was not detected within a distal region 5 kb upstream of the start site within the *p21* promoter (Fig. 7D, p21 NEG PCR; Fig. 7E). Nevertheless, we found RNA Pol II poised to initiate transcription to similar extents in cells that have been treated with HU, dauno, or a combination of both drugs. Taken together, these results indicate that impaired *p21* transcription in response to HU treatment was most likely regulated through a postinitiation mechanism.

HU suppresses p21 transcription by inhibiting RNA polymerase II elongation. To determine if transcriptional elongation was affected in cells treated with HU, ChIP assays were performed to examine RNA Pol II occupancy downstream within the p21 gene (Fig. 5A shows key regions within the *p21* gene that were examined). Indeed, the presence of RNA Pol II was decreased further downstream within Exon 2 (+5.5 kb) in cells treated with HU than in samples from daunorubicin-treated cells (Fig. 8A and C). By contrast, when the *PIG3* gene was examined, there were no significant differences in the amount of RNA polymerase detected within the transcribed region of the *PIG3* second Exon (+2.2 kb) (Fig. 8B and C). Hence, RNA Pol II is less efficient at traversing the *p21* gene after HU treatment than it is in samples from dauno-treated cells. Since the level of Pol II found at exon 2 of the PIG3 gene was quite high prior to drug treatment (Fig. 8B, lane 1 compared to lanes 2 to 4), we wanted to extend this observation by examining Pol II occupancy at regions further downstream within the *p21* and *PIG3* genes. When Pol II levels were examined at exon 3 of the p21 (Fig. 8D, +7.5 kb) or PIG3 (Fig. 8E, +5.5 kb) gene, the results were essentially the same as they were for exon 2 of these genes, except that a twofold increase in Pol II occupancy was observed at PIG3

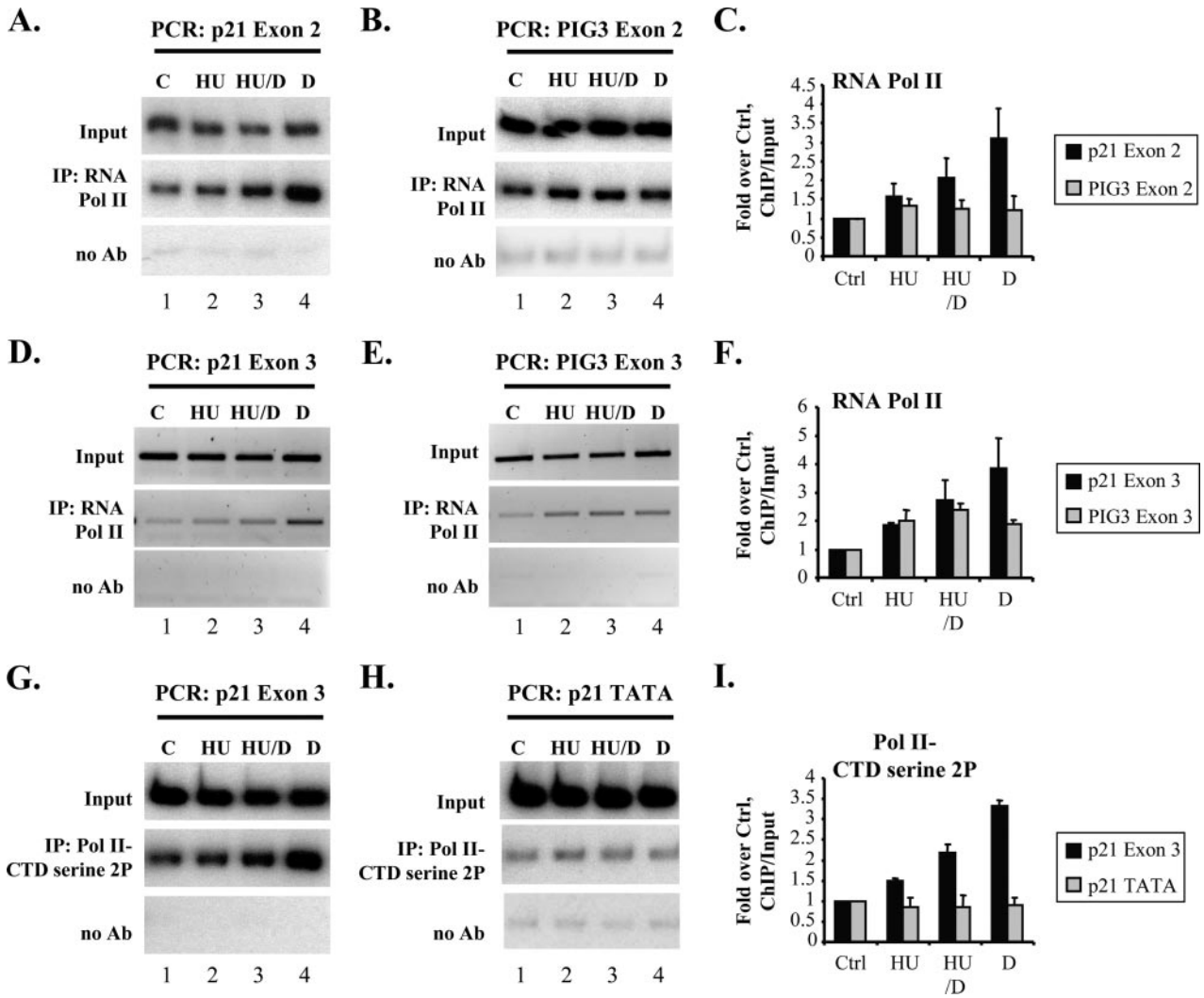

FIG. 8. HU treatment suppresses RNA polymerase II elongation of the *p21* gene. (A to F) DNA samples from the RNA Pol II ChIPs in Fig. 7C to E (RKO cells) were also subjected to PCR using primers for *p21* Exon 2 (A, p21 Exon 2) as well as the second exon within the *PIG3* gene (B, PIG3 Exon2). (C) Graphical representation of three independent experiments as shown in panels A and B. The same DNA was then subjected to PCR using primers generated within the third exon of *p21* (D, p21 Exon 3) or *PIG3* (E, PIG3 Exon 3). (F) Graphical representation of the averages for three or two experiments, shown in panels D and E, respectively. (G) ChIP assays were performed as described in Materials and Methods, using a phosphospecific antibody that recognizes phosphorylated serine 2 within the RNA Pol II CTD (anti-Pol II-CTD serine 2P), and PCRs were carried out using primers to amplify exon 3 of the *p21* gene. (H) DNA samples generated (panel G) were then used in PCRs containing primers against the TATA region of the *p21* gene as a negative control. (I) Graphical representation of the data shown in panels G and H. Data are the averages for three independent experiments. ChIP data are normalized to their respective inputs and expressed as occupancy (*n*-fold) compared to untreated RKO cells. PCRs were carried out in the presence of [α-^{32}P]dCTP in all cases except for those shown in panels D and E, in which radioactive nucleotides were excluded from the reaction mixture. For these experiments, amplicons were resolved by agarose gel electrophoresis. In all graphs, error bars represent one standard deviation. C or Ctrl, control, D, dauno; HU/D, HU and dauno.

exon 3 following drug treatment and, again, there was no significant change seen after HU or daunorubicin treatment (Fig. 8E, compare lane 1 to lane 2 to 4; Fig. 8F). It was noted that occupancy of RNA Pol II in exon 2 and exon 3 of the *p21* gene was somewhat higher in cells treated with the HU/dauno drug combination than that seen with HU alone, indicating that the presence of dauno leads to a partial rescue of transcriptional impairment. These data are consistent with our RT-PCR data (Fig. 2B, compare lanes 3 and 5 to lane 4) and suggests that the small amount of increased p21 mRNA seen with the combination of both treatments can be attributed to RNA Pol II elongation within the *p21* gene.

A substantial amount of regulation of RNA Pol II activity occurs through posttranslational modification of the large, catalytic subunit of the complex. The CTD of this subunit contains multiple (52 in human RNA Pol II) repeats of a motif (YSPTSPS) that can be phosphorylated at different sites to either maintain poised RNA polymerase at the initiation site (serine 5) or activate RNA polymerase for transcription elongation (serine 2; hereafter referred to as Pol II-CTD serine 2P) (43, 64). Gomes et al. (27) reported that doxorubicin treatment

leads to a marked increase in the amount of Pol II-CTD serine 2P within the *p21* gene locus at regions far downstream of the start site. We confirmed and extended this observation using antibodies against Pol II-CTD serine 2P to show that dauno treatment increased levels of this form of RNA Pol II within exon 3 of the *p21* gene (Fig. 8G). Relative to cells treated with daunorubicin, there was a significant decrease in the amount of Pol II-CTD serine 2P present in this region after HU treatment (Fig. 8G, lanes 2 and 4; Fig. 8I). By contrast, there was less Pol II-CTD serine 2P at the TATA box, and no differences were seen for HU- and daunorubicin-treated cells at this site (Fig. 8H and I). These data indicate that *p21* transcriptional impairment in our system is the result of reduced elongation of the *p21* gene by RNA Pol II and points to a postinitiation mechanism that is regulated by signals occurring as a result of blocked DNA replication.

DISCUSSION

Upon stalled DNA replication caused by HU, p53 protein can accumulate, bind to the promoters of its target genes, facilitate promoter histone acetylation, and recruit key components of the basal transcriptional machinery. Under the same conditions, however, transcription of the *p21* gene is substantially reduced compared to other forms of stress, such as daunorubicin, and this reduction correlates with decreased elongation of RNA Pol II along the *p21* gene. These observations have some interesting implications, which are discussed below.

It was unexpected that HU treatment leads to high levels of stabilized p53 that can bind efficiently to its some of its target genes without a subsequent increase in their transactivation. A few previous reports, however, document the accumulation of a wild-type protein that is incapable of fully activating transcription. Hypoxic conditions cause p53 levels to rise but selectively affect its posttranslational modifications and ability to regulate transcription (35, 44). Relevant to our findings is the observation that hypoxia also elicits an S-phase arrest and activation of ATR (34). Another drug shown to produce high levels of inactive p53 is 5,10-dideazatetrahydrofolate, an inhibitor of de novo purine synthesis. Here, too, p53 is unable to transcribe the *p21* gene, although in this case decreased transcription is associated with a lack of complete p53 phosphorylation and acetylation as well as inadequate histone acetylation (8). Although we cannot address whether all modifications of p53 were similarly affected after HU and dauno treatment, p53 is phosphorylated at S15, S46, and S392 and acetylated at K382 to similar extents after gamma irradiation and HU treatment (32). Therefore, we do not think posttranslational modification of p53 is likely to explain the defect seen after HU treatment, even though we found that levels of p53 generally reflect the extent of its promoter binding, as others have also reported (38, 67).

It was initially unanticipated that acetylation of histones H3 and H4 was markedly and equivalently increased by HU and daunorubicin. In addition to the link between p53 and histone acetylation, p53 has been shown to activate transcription through its association with histone methyltransferases, such as CARM1, PRMT1 (1), and SET9 (13), and with ubiquitin-conjugating enzymes, such as the hBRE1 histone E3-ubiquitin ligase (41), and p53 has been shown to repress at least one gene by mediating dimethylation of lysine 9 of histone H3 (55). Although we were unable to detect any deficiencies in histone acetylation within the *p21* gene that would explain HU-induced transcriptional impairment and we cannot rule out the possibility that other changes in histone modifications may be at play, preliminary experiments did not indicate that altered methylation of histone H3 lysines explains reduced *p21* transcription (data not shown). In light of our findings that impairment of *p21* expression is occurring downstream of transcription initiation, it is not surprising that these modifications are unaffected by HU treatment.

Our data indicate that one or more components involved in the complex process of RNA elongation are affected by agents, such as hydroxyurea, that produce a DNA replication checkpoint. Transcriptional elongation involves numerous factors and complexes, including elongins, ELL proteins, TFIIH, p-TEFb, DSIF, NELF, FACT, and many others, that play roles such as direct stimulation of elongation, alleviation of transcriptional pausing, and chromatin remodeling to facilitate elongation through chromatin (64). Although the mechanisms of transcriptional elongation are not entirely understood, recent work has revealed several levels of complexity and mechanisms that p53 could regulate. In fact, it was shown that p53 interacts with several polypeptide components of the basal transcription factor TFIIH (42, 46, 76), which has been shown to be involved not only in initiation but also in promoter clearance by RNA Pol II (17, 18, 28, 46). Functional and physical interactions between p53 and the RNA Pol II elongation factor ELL have also been reported (62). While both p53 and ELL work to enhance gene transcription in most situations, the proteins functionally antagonize each other when they are physically associated and can lead to decreased transcription of *p21* (62). The *ELL* gene is frequently involved in a chromosomal translocation which fuses a large portion of the gene to the *trithorax*-like *MLL* gene, generating the MLL-ELL protein, which is often found in acute myeloid leukemias (68) and which was also found to associate with and downregulate p53 (74). Considering the historical evidence for the success of HU in the treatment of leukemia, it is possible that HU affects p21 transcriptional elongation through the p53-ELL fusion axis. Finally, a recent study reported that p53 is involved in a feedback loop with cdk9 (a subunit of the p-TEFb elongation factor), in which p53 interacts with and is phosphorylated on S392 by cdk9 (14). However, since we previously found that p53 is equivalently phosphorylated at S392 after HU treatment and gamma irradiation, which have opposing effects on *p21* transcription, it is unlikely that cdk9 has a direct impact on p53 in our system (32). Although we do not yet know whether stalled DNA replication is regulating events at the promoter clearance stage or at a point within the elongation phase of the transcription cycle, one can imagine many scenarios in which stalled replication forks could signal to p53 or its associated elongation factor and alter gene expression.

Looking ahead, we have identified two critical directions to follow. The first is to identify those RNA elongation targets that are affected by blocking DNA replication. Particularly relevant to our observations is a recent elegant study from Gomes et al. (27), who showed that RNA Pol II, normally present and paused at the *p21* promoter, initiates transcription

in a p53-dependent manner upon stress caused by doxorubicin (an agent similar to the dauno used in our experiments). As RNA Pol II transits the *p21* gene, it becomes more phosphorylated at serine 2 of its CTD, consistent with previous studies linking this phosphorylation event to actively transcribing polymerase (43, 64). Gomes et al. also showed that a number of elongation factors, including the aforementioned ones, are recruited during this process (27). It will be interesting to determine if the functions of these are affected by stalled DNA replication and, if so, the mechanism by which this occurs. The second direction is to determine the signaling pathway that affects the process of elongation of p21 RNA transcripts. The upstream responders to stalled DNA replication caused by either depleted dNTPs after HU treatment or blocked DNA elongation by aphidicolin include RP-A, H2AX, ATR-ATRIP, claspin, and the repair clamp and clamp-loader complexes, Rad 17 and 9-1-1 (6, 31). Activated Chk1, the mediator kinase that is a key target of this complex, may regulate one or more of the elongation factors that are involved in transcription of the *p21* gene. It is hoped that future experiments will shed light on these questions.

It is fascinating to ask why, when *p21* is so regulated, other p53 targets, such as *PIG3*, are not affected by this process. Even though RNA Pol II was shown by Espinosa and Emerson (23) to be present and poised at a subset of p53 targets, including *p21*, prior to p53 activation, others, such as *PIG3*, do not have much preloaded RNA polymerase II. These authors also found that once p53 is induced, there is a net loss of RNA Pol II at the *p21* initiation site at early time points, while in at least one case (*FAS*/*Apo1*), there is no such loss at the *FAS*/*Apo1* promoter, suggesting that the latter has a much higher rate of reinitiation that could conceivably overcome a reduced rate of elongation at this gene. Whether this is the case with *PIG3* remains to be determined. Alternately, poised RNA polymerase at the *p21* TATA region may recruit one or more distinct elongation factors that are not involved in transcription of other genes, such as *PIG3*, and such factors are the direct targets of the DNA replication checkpoint.

It should be mentioned here that our results are not seen in every human cell line; p21 mRNA accumulates in HU-treated MCF7 cells that contain wild-type p53 (54; M. Mattia and C. Prives, unpublished observations). Nevertheless, they are not unique to RKO cells, since we also observed that markedly less p21 mRNA accumulated after HU treatment than after daunorubicin treatment in another cell line (HCT116) (see Fig. S1C and D in the supplemental material). It will be interesting to eventually determine the reasons for cell-type differences in responses to stalled DNA replication. As we understand more about the upstream signaling pathways and their relationships to factors that regulate transcription, such differences should be elucidated. Clearly, there is much to do to further understand the process by which some transcription units are differentially affected when DNA replication is stalled.

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