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Repression of *CDK1* and Other Genes with CDE and CHR Promoter Elements during DNA Damage-Induced G_2/M Arrest in Human Cells

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Entry into mitosis is controlled by the cyclin-dependent kinase CDK1 and can be delayed in response to DNA damage. In some systems, such G_2/M arrest has been shown to reflect the stabilization of inhibitory phosphorylation sites on CDK1. In human cells, full G_2 arrest appears to involve additional mechanisms. We describe here the prolonged (>6 day) downregulation of CDK1 protein and mRNA levels following DNA damage in human cells. This silencing of gene expression is observed in primary human fibroblasts and in two cell lines with functional p53 but not in HeLa cells, where p53 is inactive. Silencing is accompanied by the accumulation of cells in G_2, when CDK1 expression is normally maximal. The response is impaired by mutations in *cis*-acting elements (CDE and CHR) in the CDK1 promoter, indicating that silencing occurs at the transcriptional level. These elements have previously been implicated in the repression of transcription during G_1 that is normally lifted as cells progress into S and G_2. Interestingly, we find that other genes, including those for CDC25C, cyclin A2, cyclin B1, CENP-A, and topoisomerase IIα, that are normally expressed preferentially in G_2 and whose promoter regions include putative CDE and CHR elements are also downregulated in response to DNA damage. These data, together with those of other groups, support the existence of a p53-dependent, DNA damage-activated pathway leading to CHR- and CDE-mediated transcriptional repression of various G_2-specific genes. This pathway may be required for sustained periods of G_2 arrest following DNA damage.

The cell cycle consists of alternating S phases and mitoses that must be carefully controlled to ensure that genomic integrity is maintained. For example, a failure to complete DNA synthesis before entering mitosis or to complete mitosis before entering S phase can lead to aneuploidy or cell death. The cell cycle is also sensitive to DNA damage, presumably so that repair processes have sufficient time to operate. Failure to repair DNA damage before it is transmitted to daughter chromosomes or to daughter cells results in the accumulation of mutations, increasing the likelihood of tumorigenesis. Surveillance mechanisms, or checkpoints, exist that detect DNA damage or problems in completing specific cell cycle events and inhibit cell cycle progression at appropriate points (16, 17). In many systems, the checkpoints have been shown to work, at least partly, by influencing the activity of cyclin-dependent kinases (CDKs) (13).

In response to DNA damage, mammalian cells can arrest at both the G_1/S and the G_2/M transitions. Key checkpoint targets in G_1/S arrest are thought to be the G_1 CDKs (CDK2, CDK4, and CDK6), which activate the G_1/S transition by phosphorylating RB, thereby releasing the E2F transcription factors which promote the transcription of genes required for the transition (7, 14). DNA damage activates p53, which transcribes the gene for p21, a G_1 CDK inhibitor. Correspondingly, a key checkpoint target in G_2/M arrest is CDK1 (also called CDC2), which promotes entry into mitosis, provided it is bound to cyclin B1 and dephosphorylated at Tyr15 and Thr14. Thus, based on work with mammalian cells and fission yeast, a DNA damage-induced pathway leading to the inhibitory phosphorylation of CDK1 has been proposed (for reviews, see references 39, 46, 55, and 57). In this pathway, DNA damage activates ATM kinase, which activates Chk1 or Chk2 (36) kinases which, in turn, phosphorylate CDC25C, the CDK1-activating phosphatase. Phosphorylated CDC25C is bound and sequestered by a p53-inducible 14-3-3 protein and therefore is unable to activate CDK1. Chk1 kinase may also activate the CDK1-activating kinase Wee 1 (41).

Although inhibitory phosphorylation of CDK1 clearly follows DNA damage in mammalian cells (20, 21, 31, 42, 44), it is not a universal eukaryotic mechanism for DNA damage-induced G_2/M arrest (2, 51, 61), and there is evidence that other mechanisms are involved. Thus, radiation-induced G_2/M arrest is only partly suppressed in human cells expressing mutant CDK1 that cannot be phosphorylated at Tyr15 and Thr14 (27), and G_2/M-arrested cells have been described in which endogenous CDK1 is dephosphorylated at Tyr15 and Thr14 (59). Furthermore, recent data suggest that control of cyclin B1 nuclear localization (28) and action of the CDK inhibitor p21 (12) may also be important mechanisms for G_2/M arrest in human cells. Mammalian cells therefore use multiple mechanisms for effecting DNA damage-induced G_2/M arrest, and it seems likely that further pathways will be uncovered before such G_2/M arrest is fully understood.

Our interest in the role of CDK1 in the cellular response to DNA damage stems from studies of a human cell line (HT2-19) in which endogenous *CDK1* gene expression is dependent on the presence of an inducer isopropyl-β-D-thiogalactopyranoside (IPTG) in the growth medium (25). In the absence of

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IPTG, HT2-19 cells accumulate transiently in G_2 and undergo apoptosis or repeated S phases without intervening mitoses (rereplication). The latter phenotype suggested that CDK1 is required to prevent the initiation of S phase before G_2 and mitosis have been completed, a surveillance mechanism first described for fission yeast (18). The present study arose from our observation that a very similar rereplicative phenotype is observed in parental HT1080 cells following DNA damage, consistent with the notion that CDK1 activity is downregulated in response to DNA damage. In confirming and characterizing such downregulation, we appear to have identified a general DNA damage-induced signaling pathway in human cells, culminating in the continued transcriptional repression of a family of genes that are normally derepressed as cells progress from G_1 into S and G_2.

MATERIALS AND METHODS

Cell culture and irradiation. HT1080 (fibrosarcoma), HT2-19, and HeLa (cervical carcinoma) cells were used as previously described (25, 45). HCT116 (colon carcinoma) and WI-38 (primary fibroblasts) cells were obtained from the American Type Culture Collection. Cells were grown in Dulbecco's modified essential medium (GIBCO) supplemented with 10% fetal calf serum, penicillin (80 U/ml), and streptomycin (80 mg/ml) and maintained at 37°C in a humidified atmosphere of 5% CO_2. Cells were irradiated, after having been allowed to attach and grow overnight, in an IBL 637 ^{137}Cs irradiator (CIS BIO International) delivering 1.85 to 20 Gy/min, and samples were harvested at various times after irradiation. A dose of 6 Gy was used for all cells except HeLa cells, because this dose gave an accessible population of G_2/M-arrested or rereplicating cells; while <10% of HT1080 (43, 62), HCT116 (54), or WI-38 (38) cells recover from this dose, sufficient G_2/M-arrested cells remain attached to the plate for analysis. A lower dose (3 Gy) was required for HeLa cells because at 6 Gy, most cells become detached and are lost, presumably by apoptosis, within 1 to 2 days. To arrest WI-38 cells in G_0/G_1, the medium of confluent plates was replaced with serum-free medium for 7 days. Release from G_0/G_1 arrest was achieved by trypsinizing cells and replating them at a low density in medium with 20% fetal calf serum.

Flow cytometry. Propidium iodide (PI)-stained nuclei were prepared by modification of a previously described method (25, 40). Briefly, about 5×10^5 cells were centrifuged, and the pellet was resuspended in 1 ml of solution I (10 mM NaCl, 1 mg of trisodium citrate per ml, 0.06% [vol/vol] Nonidet P-40, 25 μg of PI per ml, 10 μg of RNase A per ml) and incubated at room temperature for 30 min. One milliliter of solution II (1.5% [wt/vol] citric acid, 0.25 M sucrose, 40 μg of PI per ml) was added. The nuclear suspension was agitated and stored at 4°C overnight before flow cytometric analysis on a Becton Dickinson FACScan. CellQuest software (Becton Dickinson) was used for acquisition and manipulation of the data.

Plasmids and in vitro mutagenesis. Plasmid pWTCDK1-LUC contains 3 kb of the promoter of the human *CDK1* gene upstream of a luciferase reporter gene and the *bla* and *gpt* genes as selection markers for use, respectively, in bacteria and mammalian cells. To make pWTCDK1-LUC, the CDK1 cDNA was removed from pCDC/gpt (25) by digestion with *Not*I to generate a 9-kb *Not*I fragment, which was purified, end filled, and ligated to an end-filled 2.7-kb *Bam*HI/*Hin*dIII fragment from pGL2-B (Promega). A mutagenesis kit (QuickChange Site-Directed; Stratagene) was used to obtain all the mutations in the *CDK1* promoter. Briefly, a 225-bp fragment of the *CDK1* promoter containing the CDE and CHR elements was cloned into pBluescript II KS(+) (Promega) for mutagenesis. The following primers, and complementary primers, were used to create mutations 1 to 5 (CDE and CHR are shown in italic type, and the mutations are shown in boldface type): M1CDK1-LUC (5′-GGGGCCCTTTAG*GCGC**T**G*TGAGT*TTGAA*ACTG-3′), M2CDK1-LUC (5′-GGGGCCCTTTAG*C**T**CGG*TGAGT*TTGAA*ACTGCTCGCAC-3′), M3CDK1-LUC (5′-GGGGCCCTTTAG*A**TATT***TGAGT*TTGAA*ACTGCTCGCACTTGGCTTC-3′), M4CDK1-LUC (5′-GGGGCCCTTTAG*CGCGG*TGAGT*TT**T**AA*ACTGCTCGCAC-3′), and M5CDK1-LUC (5′-GGGGCCCTTTAG*CGCGG*TGAGT***GGTCC***CACTGCTCGCACTTGGCTTC-3′). The modified *Xba*I/*Xma*I fragments were sequenced to confirm that only the desired mutation had been introduced and cloned back into the *Xba*I/*Xma*I sites of pWTCDK1-LUC to generate pM1CDK1-LUC, pM2CDK1-LUC, and so forth.

Transfections. For stable transfections, a Gene Pulser (Bio-Rad) was used as previously described (25). pWTCDK1-LUC (8 μg) or a mutated derivative was linearized at a unique *Sal*I site and mixed with 3.5×10^6 cells before electroporation. To select for colonies stably transfected with *gpt*, 10 μg of mycophenolic acid per ml and 100 μg of xanthine per ml were was added to the medium 48 h after electroporation. Colonies appeared after 10 to 14 days in selective medium. A pool of at least 50 colonies was collected for each construct. Transient transfections were performed with LipoTAXI (Stratagene). Briefly, 2×10^5 cells were plated in a 35-mm dish 24 h before transfection. pWTCDK1-LUC or a mutated derivative (2 μg) and pRLCMV (14 ng; Promega) were mixed with 20 μl of LipoTAXI and 280 μl of serum-free, antibiotic-free medium and kept for 30 min at room temperature. This mixture was added to the cell culture and incubated for 6 h. The mixture was then removed and replaced with fresh complete medium. Cells were incubated overnight, trypsinized, and split in two. One of the cell suspensions was irradiated, and both were distributed into two 35-mm plates; irradiated and control cells were harvested for luciferase assays 24 and 48 h later.

Western blots. Standard protocols were used as previously described (25). Briefly, cell lysates were prepared in Laemmli buffer and separated on a sodium dodecyl sulfate–10% polyacrylamide gel. The proteins were transferred onto an Immobilon P membrane (Millipore) and probed with an anti-CDK1 monoclonal antibody (Santa Cruz Biotechnology, Inc.) raised against the complete CDK1 molecule or a rabbit polyclonal antibody raised against the actin protein (Sigma). Specific proteins were visualized with an ECL detection system (Amersham). Horseradish peroxidase-conjugated goat anti-mouse (PO447; DAKO) or goat anti-rabbit (P448; DAKO) immunoglobulins were used as secondary antibodies.

Histone H1 kinase assays. Immunoprecipitation of cell lysates with an antibody to CDK1 and assays of precipitates for histone H1 kinase activity were performed as described previously (25).

RT-PCR. Cells (3×10^6 per 15-cm plate) were grown overnight (16 h), irradiated, and lysed in guanidinium thiocyanate immediately or 1, 3, or 6 days later for the preparation of RNA by density gradient centrifugation (47). RNA was reverse transcribed using a reverse transcriptase (RT) system (Promega) according to the manufacturer's instructions, and 1 μl of reverse-transcribed RNA, undiluted or diluted in water, was used for each PCR.

Reagents and conditions for the semiquantitative analysis of CDK1 transcripts have been described previously (25). Briefly, separate reactions specific for CDK1 cDNA and a reference cDNA phosphoglycerate kinase (PGK) were carried out at low cycle numbers (approximately 20) to ensure presaturation conditions. Products were visualized by Southern analysis and quantitated on a PhosphorImager (Molecular Dynamics), normalizing the CDK1 signal to the PGK signal.

Important variables for the remaining PCRs used in this report are shown in Table 1. Concentrations of primers, *Taq* polymerase, and buffer components other than $MgCl_2$ were as described previously (25). In these assays, presaturation conditions were achieved by serial dilutions of the cDNA templates. Unless stated otherwise, all temperature treatments were the same: 35 cycles (30 for topoisomerase IIα) were preceded by incubation at 94°C for 3 min and followed by incubation at 72°C for 5 min. One amplification cycle consisted of 30 s at 94°C, 30 s at 65°C, and 30 s at 72°C. Following agarose gel electrophoresis, products were visualized by staining with ethidium bromide.

Luciferase assays. Cells were assayed for luciferase activity with a 1253 BioOrbit luminometer and a Dual-Luciferase Reporter Assay System (Promega). Briefly, one 3.5-cm plate of transiently transfected cells was used per time point; the cells were harvested in 0.5 ml of lysis buffer. To assay firefly luciferase activity (expression driven by the *CDK1* promoter), luminosity was measured after 20 μl of lysate was mixed with 100 μl of luciferase assay reagent II. Then, to assay *Renilla* luciferase activity (from control vector pRLCMV), luminosity was measured again after the addition of 100 μl of Stop and Glo reagent (Promega). The activities of the experimental reporter (firefly luciferase) were normalized to the activities of the internal control reporter (*Renilla* luciferase).

Statistical methods. Luciferase data from four identical transient transfection assays were analyzed by comparing each mutation with the control (wild-type) group using modified *t* tests and a significance criterion determined by Dunnett's procedure. The *t* tests were modified in that a single pooled estimate of the standard error which contained data from all six groups was used. Dunnett's procedure is a method of keeping the false-positive (type I) error rate within a certain level for a set of pairwise comparisons as a whole. Dunnett's procedure indicated for five pairwise comparisons with the control group and four observations in each group that the use of a significance criterion of greater than 2.90 will keep the overall false-positive rate to within 5% and that a value of greater than 3.29 will keep the overall false-positive rate to within 1%. The *t* values calculated for mutations 1 to 5 were 2.44, 0.53, 4.56, 0.03, and 4.54, respectively. These values indicated that mutations 3 and 5 behave differently from the control at the 1% significance level.

Nuclear extracts and electrophoretic mobility shift assays. Preparation of nuclear extracts (11) and binding reactions (34) were carried out at 0 to 4°C. The DNA probe and competitors were prepared by annealing complementary oligonucleotide pairs. The wild-type pair was 5′-GTTTAGCGCGGTGAGTTTGAAACTGC-3′ and 5′-GGCAGTTTCAAACTCACCGCGCTAAA-3′. The mutant M3 pair was 5′-GTTTAGATATTTGAGTTTGAAACTGC-3′ and 5′-GGCAGTTTCAAACTCAAATATCTAAA-3′. The unrelated pair was 5′-GGCGAACAGTAGCTTCCTGCTCCGCT-3′ and 5′-GAGCGGAGCAGGAAGCTACTGTTCGC-3′. The probe (~0.1 μCi/ng) was labeled by an end-filling reaction in the presence of [α-^{32}P]dCTP. Binding reactions (21 μl) contained nuclear extract (5 to 10 μg), Tris-HCl (pH 8.0) (36.5 mM), sodium deoxycholate (0.58%), glycerol (10.7%), NaCl (113 mM), $MgCl_2$ (0.4 mM), EDTA (0.2 mM), NaF (1.35 mM), dithiothreitol (1.5 mM), protease inhibitor cocktail (Complete; Boehringer Mannheim) poly(dA-dT) (1 μg), an unrelated oligonucleotide pair (100 ng), NP-40 (1.5%), labeled probe (0.1 ng) and, when needed, a competitor oligonucleotide pair (50 ng). Reactions were resolved on a 6% polyacrylamide gel in

TABLE 1. PCR primers used in this study

Gene	Primers[a]	$MgCl_2$ (mM)	Product size (bp)
CDC25C	5′-TATCTGGGAGGACACATCCAGG-3′ (S) 5′-CAAGTTGGTAGCCTGTTGGTTTG-3′ (A)	2.0	552
Cyclin A	5′-CAGCCTGCAAACTGCAAAGTTG-3′ (S) 5′-TTTAGTGTCTCTCTGGTGGGTTGAGG-3′ (A)	2.0	326
Topoisomerase IIα	5′-TGTCGTGTCAGACCTTGAAGCTG-3′ (S) 5′-CCCCCTTGGATTTCTTGCTTG-3′ (A)	4.0	359
CENP-A	5′-AGAAGCCAGCCTTTCGCTC-3′ (S) 5′-AATTGAAGTCCACACCAC-3′ (A)	2.0	337
Cyclin B1	5′-TCTACCTTTGCACTTCCTTCGG-3′ (S) 5′-TACACCTTTGCCACAGCCTTGG-3′ (A)	2.0	402
Cyclin E	5′-TACAGATTGCAGAGCTGTTGGATC-3′ (S) 5′-AAAAGCAAACGCACGCCTCC-3′ (A)	3.5	494
CDK2	5′-GCTCACCCTTTCTTCCAGGATG-3′ (S) 5′-TGGTACGGCAAATCTAACGTGTAG-3′ (A)	3.5	373
Cyclin D1	5′-AAGATGAAGGAGACCATCCCCCTG-3′ (S) 5′-ATCACTCTGGAGAGGAAGCGTGTG-3′ (A)	2.0	377
PGK	5′-CCTCCGCTTTCATGTGGAGGAAGA-3′ (S) 5′-GTAAAAGCCATTCCACCACCAA-3′ (A)	2.0	360
Luciferase	5′-ATTCTTCGCCAAAAGCACTCTG-3′ (S) 5′-GAAGTGTTCGTCTTCGTCCCAG-3′ (A)	2.0	418

[a] S, sense; A, antisense.

0.5× Tris-borate-EDTA at 12 V/cm for 90 min. The gel was fixed, dried, and analyzed with a PhosphorImager.

RESULTS

DNA damage in HT1080 cells causes downregulation of CDK1 kinase activity and rereplication. We were interested to know if the rereplication observed in HT2-19 cells after the repression of endogenous *CDK1* gene expression required a loss of CDK1 kinase activity or a loss of the CDK1 protein itself. Because DNA damage has been shown to downregulate CDK1 kinase activity in some systems by preventing stimulatory dephosphorylation, we exposed parental HT1080 cells to the topoisomerase II inhibitor mitoxantrone, a treatment known to generate double-stranded breaks in DNA. This treatment caused the downregulation of CDK1 kinase activity and rereplication very similar to that observed in HT2-19 cells following the removal of IPTG (Fig. 1). A similar rereplicative phenotype was induced by ionizing irradiation (see below), which also induces double-stranded breaks.

CDK1 protein levels fall after DNA damage in human cells. If inhibitory phosphorylation and/or subcellular relocalization is solely responsible for the loss of CDK1 kinase activity following DNA damage, CDK1 protein levels should be unaffected by DNA-damaging agents. To test this notion, Western analysis was carried out and revealed, unexpectedly, that CDK1 protein levels were downregulated in HT1080 cells following gamma irradiation (Fig. 2). This observation kept open the possibility that a loss of the CDK1 protein itself is required for rereplication to occur. More importantly, it suggested that a signaling pathway for downregulating CDK1 protein levels in response to DNA damage must exist in human cells. In principle, such a pathway could play an important role in establishing an effective G_2 delay in response to DNA damage. To determine whether the response could be detected in human cells other than HT1080, we carried out Western analyses on three other sources of human cells: a colon carcinoma cell line (HCT116), a cervical carcinoma cell line (HeLa), and primary

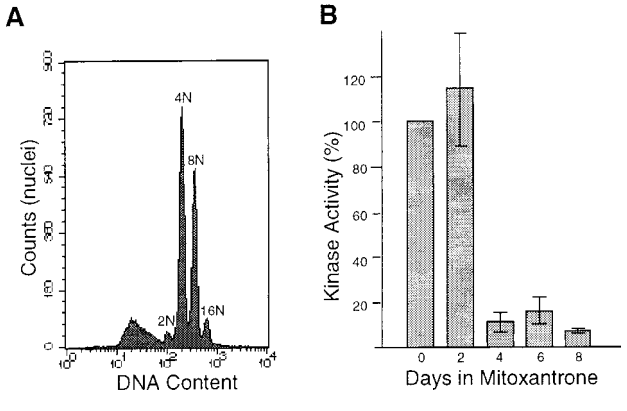

FIG. 1. DNA rereplication and downregulation of CDK1 kinase activity after DNA damage in HT1080 cells. (A) Flow cytometric analysis of DNA content in nuclei of HT1080 cells 6 days after the addition of 25 ng of mitoxantrone per ml. The number of haploid genome equivalents (2N, 4N, etc.) is indicated for each peak. (B) CDK1 histone H1 kinase activity in immunoprecipitates of HT1080 cell extracts prepared at the indicated times after the addition of 25 ng of mitoxantrone per ml. The average ± standard deviation for triplicate assays is shown.

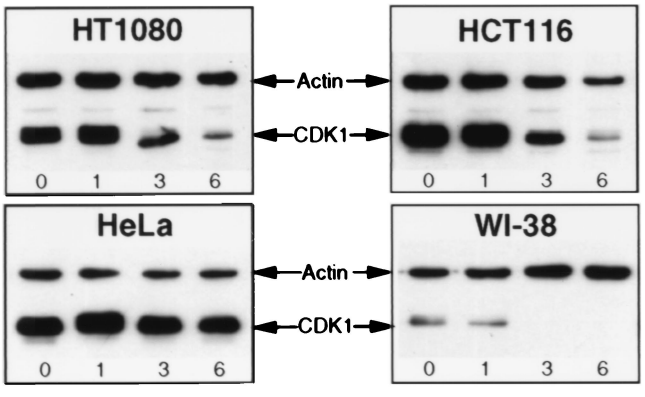

FIG. 2. Detection of CDK1 by Western analyses of the indicated cell lines. Cell extracts were prepared at the indicated times (days) after gamma irradiation at 6 Gy (or 3 Gy for HeLa cells). Duplicate samples were analyzed for actin content.

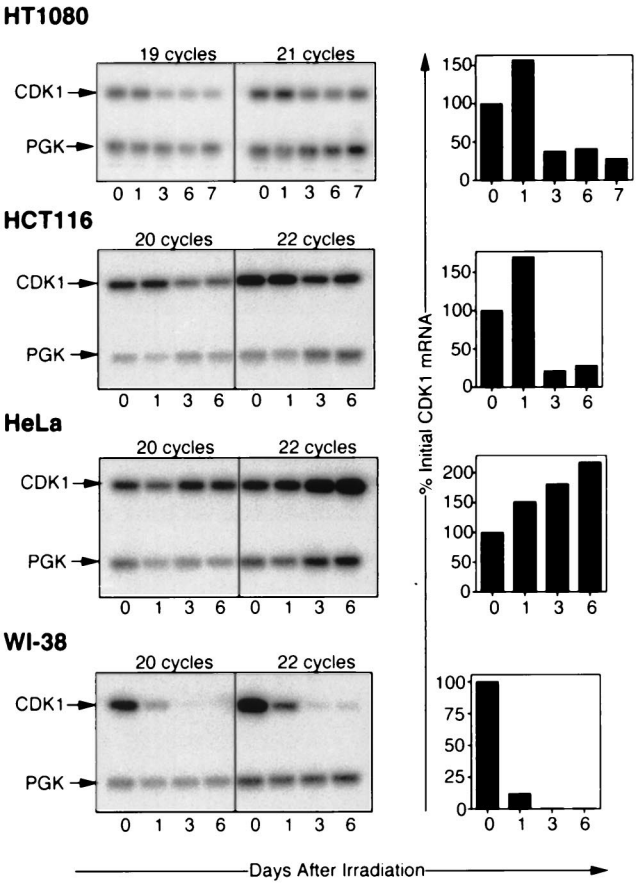

FIG. 3. Semiquantitative RT-PCR assays for steady-state CDK1 mRNA levels in cells harvested at the indicated number of days after gamma irradiation at 6 Gy. (Left panels) RT-PCR products hybridizing to probes specific for CDK1 or PGK after two different numbers of PCR cycles. (Right panels) Histograms showing amounts of CDK1-specific products, measured as described in Materials and Methods, at the indicated times.

human fibroblasts (WI-38). Interestingly, the most rapid and pronounced downregulation of CDK1 protein levels was observed in WI-38 cells, while HCT116 cells displayed a response similar to that detected in HT1080 cells and HeLa cells showed no response (Fig. 2).

***CDK1* transcription is repressed in response to DNA damage.** We wanted to know whether the loss of CDK1 reflected an increase in CDK1 degradation or a decrease in *CDK1* gene expression in response to DNA damage. We therefore assayed for CDK1 mRNA in RNA taken from cells at various times after irradiation by RT-PCR. In this way, CDK1 mRNA downregulation was detected (Fig. 3) and seen to correlate well with the CDK1 protein downregulation shown in Fig. 2. Thus, downregulation was again most rapid and pronounced in WI-38 cells, intermediate in HT1080 and HCT116 cells, and absent in HeLa cells. Downregulation of CDK1 mRNA could reflect increased degradation or decreased synthesis of CDK1 mRNA. The latter mechanism is supported by our observation that HT1080 cells transfected with a luciferase reporter gene fused to the *CDK1* promoter downregulate luciferase in response to DNA-damaging agents (see below).

Transcriptional repression cannot be explained by accumulation in G_1. *CDK1* transcription is known to be minimal in G_1 and to become active as cells move into S phase (8, 37, 52, 58), allowing sufficient CDK1 protein to accumulate by the time it is required for promoting mitosis in late G_2. A trivial explanation for the apparent downregulation of CDK1 mRNA in response to DNA damage could therefore be that most cells accumulate in G_1. Profiles of DNA content following DNA damage (Fig. 4) rule out this explanation. This finding is most clearly seen in WI-38 cells, which show a much greater tendency to accumulate with a 4N DNA content than with a 2N

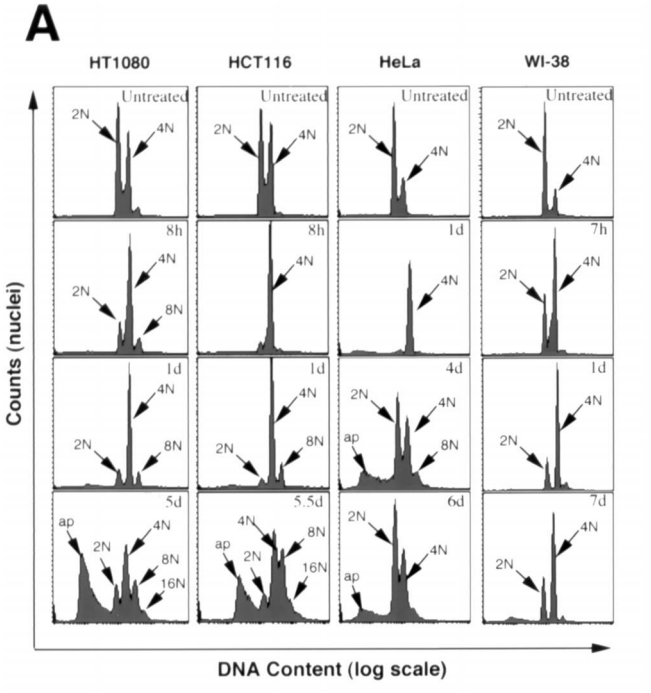

FIG. 4. (A) Flow cytometric profiles of DNA content in different human cell types used in this study. Cells were either untreated or harvested at the indicated times (d, day) after gamma irradiation. Peak haploid genome equivalents (2N, 4N, etc.) are indicated. Peaks with greater than 4N content indicate DNA rereplication, and peaks with less than 2N content (ap) probably represent apoptotic nuclei (25). (B) Duplex RT-PCR assays (lanes 5 to 8) for PGK and cyclin E transcripts in WI-38 cells that were asynchronous (lane 5), that were confluent (Conf) and in serum-free medium (LS) for 7 days (lane 6) or 11 days (lane 7), or that had been irradiated (6 Gy) 7 days prior to harvest (lane 8). Reverse transcription products were used for PCR over a range of dilutions. A dilution (1:125) which generated visible but submaximal amounts of cyclin E (E)- and PGK (P)-specific products is shown. Flow cytometry (data not shown) confirmed that the growth conditions generated cells that were asynchronous (lane 5) or enriched for 2N (lanes 6 and 7) or 4N (lane 8) content. Control lanes show products from assays with no template (lane 4) or the same template as in lane 5 but undiluted and with PGK primers only (lane 2) or cyclin E primers only (lane 3). A 123-bp DNA ladder was used as a size marker (lane 1).

DNA content. The same is true for HT1080 and HCT116 cells, although at later times the effect is accompanied by rereplication (appearance of 8N, 16N, etc., cells) and apoptosis (appearance of cells with less than 2N content). Interestingly, and in contrast to the other cells tested, HeLa cells, which did not show downregulation of CDK1 mRNA and protein, showed only a transient accumulation in G_2 1 day after irradiation; by day 6, their DNA profile was not very different from that of untreated cells. Despite the differences in profiles, none of the cells showed a net accumulation in G_1 (2N) after irradiation.

The above argument relies on the assumption that 4N cells, as detected by flow cytometry, are in G_2. In some circumstances, however, G_1 cells can have a 4N content, for example, after exiting from abortive mitosis (32). In such cases, one might expect the expression of genes normally associated with G_1, such as the cyclin E or CDK2 genes, to be upregulated. This was shown to be the case for cyclin E in 4N G_1 cells that had exited abortive mitosis (32). However, as shown in Fig. 4B (lane 8) and in controls for an experiment described later in this paper (see Fig. 8), neither cyclin E nor CDK2 transcripts accumulated in irradiated HT1080 or WI-38 cells, arguing against a net accumulation in G_1. Furthermore, the fact that there is no net loss of cyclin E transcripts after irradiation, as there is in serum-starved cells that accumulate in G_0/G_1 (Fig. 4B), argues against a net accumulation in G_0 or early G_1. We conclude that 4N cells accumulating after irradiation are genuinely in G_2.

DNA damage-induced transcriptional repression requires CDE and CHR elements. The lack of *CDK1* transcription during G_1 has been attributed to transcriptional repression mediated by *cis*-acting sequences (CDE and CHR) in the *CDK1* promoter, repression that is lifted as cells progress from G_1 into S/G_2 (34). It seemed possible that the lack of *CDK1* transcription that we observed in largely G_2 populations could be explained if DNA damage prevented the usual derepression of *CDK1* transcription that occurs when cells exit G_1 and progress into S and G_2. To test this notion, we constructed a series of plasmids in which the firefly luciferase reporter was driven by the *CDK1* promoter, which was either wild type or carried one of five mutations in CDE or CHR (Fig. 5A). Each of these was transiently cotransfected into HT1080 cells with a control plasmid in which the renilla luciferase reporter was driven by a cytomegalovirus promoter. Cells were irradiated 24 h after transfection, and luciferase activity was measured after a further 24 and 48 h. None of the *CDK1* promoters was impaired in its ability to express luciferase before irradiation (data not shown), whereas the ability to downregulate firefly luciferase between 24 and 48 h after irradiation was lost for promoters carrying mutations 3 and 5, in which the CDE and CHR elements had been completely removed (Fig. 5B).

Because the transient assays allowed analysis of downregulation only during a restricted time window, we decided to analyze the response over a longer time period with stably transfected cells. HT1080 cells were therefore stably transfected with either the wild-type construct (pWTCDK1-LUC) or the construct carrying mutation 3 (pM3CDK1-LUC) or 5 (pM5CDK1-LUC). Another construct (pSV40-LUC) with the luciferase gene under the control of the simian virus 40 minimal promoter was transfected into HT1080 cells as a control. Stable transfectants were irradiated, total RNA extracts were prepared 0, 1, 3, or 6 days later, and RT-PCR was used to measure steady-state levels of luciferase mRNA. For cells transfected with pWTCDK1-LUC, downregulation of luciferase mRNA was clearly seen by 3 days, whereas downregulation was consistently less pronounced for cells in which luciferase expression was driven by either of the mutant promoters

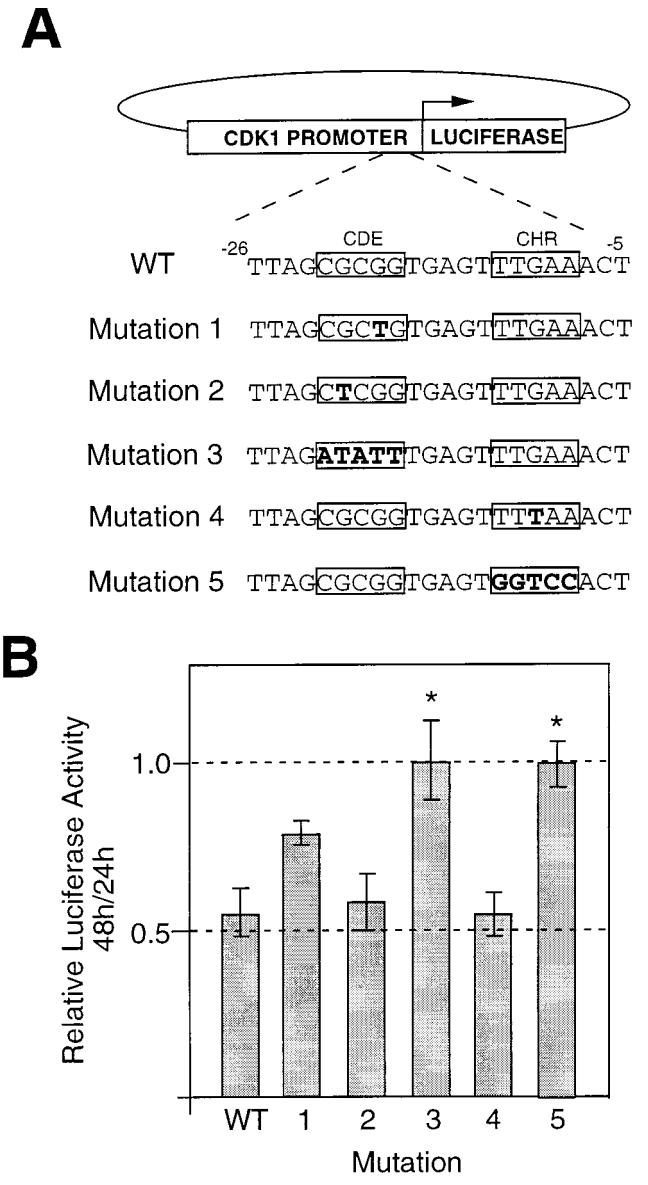

FIG. 5. Transfection assays for DNA damage-induced repression of luciferase gene transcription driven by wild-type (WT) or mutant *CDK1* promoters. (A) Diagrammatic representation of reporter constructs showing different mutations (in boldface type) engineered into the CDE or CHR elements of the *CDK1* promoter. (B) Luciferase activity at 48 h relative to 24 h after gamma irradiation (6 Gy) in HT1080 cells transiently transfected (24 h before irradiation) with the indicated reporter constructs. The values shown represent the average of four independent experiments ± the standard deviation. The asterisks mark mutations showing results significantly (P, <0.01) different from those obtained with the wild type.

(Fig. 6A). No loss of luciferase mRNA was detected in pSV40-LUC-transfected cells up to 3 days after irradiation, although a small decrease, whose significance was unclear, was detectable after a further 3 days.

In a semiquantitative analysis of luciferase downregulation, we used duplex RT-PCRs designed to detect, in the same sample, mRNAs for both luciferase and the housekeeping enzyme PGK (Fig. 6B). PGK mRNA clearly was not downregulated by irradiation. For dilutions (1:5) of reverse-transcribed RNA yielding PCR products at well below saturating levels, the intensity of the luciferase product relative to the PGK product was measured digitally. The ratio at day 0 was arbi-

FIG. 6. RT-PCR assays for luciferase transcripts in stably transfected HT1080 cells. Pools of clones transfected with the indicated plasmids were harvested at the indicated times after gamma irradiation to make RNA. (A) Assays for luciferase only with undiluted cDNA. (B) Duplex assays for luciferase and PGK with and without dilution of cDNA. Arrows indicate luciferase (L) and PGK (P) PCR products. RNA from untransfected cells was used as a control (lane C).

trarily adjusted to 1, and all other ratios were adjusted accordingly. In this way, levels of luciferase transcripts in cells transfected with pWTCDK1-LUC were estimated to fall by factors of 1.3, 5.5, and 100 at days 1, 3, and 6, respectively; the equivalent values for pM5CDK1-LUC were 1, 1.4, and 1.8.

To begin to address the molecular mechanism of CDE-mediated gene silencing following gamma irradiation, we carried out electrophoretic mobility shift assays. Nuclear extracts from WI-38 cells were incubated with a radiolabeled *CDK1* promoter probe and analyzed by nondenaturing acrylamide gel electrophoresis (Fig. 7). A mobility shift that was detected in gamma-irradiated cells was lost when an excess of unlabeled probe was included in the assay but not when a similar excess of probe mutated in its CDE element was included (Fig. 7, lanes 1 to 4). A similar shift was detected in G_0/G_1-arrested cells but not in cells that had been released from such an arrest into S phase by the readdition of serum (Fig. 7, lanes 5 to 7). These preliminary results are consistent with the possibility that the nuclear factor(s) responsible for silencing CDE- and CHR-containing promoters in G_1 also functions in G_2-arrested cells that accumulate after gamma irradiation.

Taken together, these data clearly implicate the CDE and CHR elements in the mechanism leading to the transcriptional repression of CDK1 after DNA damage and provide support for the idea that normal G_1 transcriptional repression extends into G_2 after DNA damage.

DNA damage induces downregulation of other genes carrying CDE and CHR elements in their promoters. Several genes, including those for cyclin A (33), cyclin B1 (30), CDC25C (35), CENP-A (50), and topoisomerase IIα (24), are similar to *CDK1* in being upregulated as cells progress from G_1 into S and G_2 and in having promoters with CDE and CHR elements. For the cyclin A and CDC25C genes, the CDE and CHR elements have been shown to mediate transcriptional repression during G_1. To test whether these genes share with *CDK1* the further property of being downregulated in response to DNA damage, we set up RT-PCR assays to detect their transcripts in RNA from irradiated HT1080 or WI-38 cells. As controls, we used RT-PCR assays for the cyclin E, CDK2, and cyclin D1 genes, cell cycle genes whose products are required during the G_1/S transition and therefore are expressed in G_1. As a further control, RT-PCR for the PGK gene was used. For each assay, products were diluted (1-, 5-, 25-, 125-, or 625-fold) prior to their use as PCR templates. The results for dilutions giving visible, nonsaturating amounts of PCR products are shown in Fig. 8. Of the five S/G_2-expressed genes, four (those for CDC25C, cyclin A, topoisomerase IIα, and CENP-A) were downregulated in both HT1080 and WI-38 cells. As observed for *CDK1* (Fig. 3), downregulation was more rapid and pronounced in WI-38 than in HT1080 cells. The fifth gene (cyclin B1) was clearly downregulated in WI-38 but not in HT1080 cells. The four control genes showed a constant expression level during the 6 days following irradiation. Thus, of the nine genes tested, only the five that are expressed preferentially in S/G_2 and have CDE and CHR promoter elements were downregulated in response to gamma irradiation. The expression pattern and promoter elements that these genes have in common with each other are also shared by *CDK1*, for which downregulation was shown to be transcriptional. Thus, although the data of Fig. 8 cannot rule out a postranscriptional mechanism for radiation-induced downregulation, a transcriptional repres-

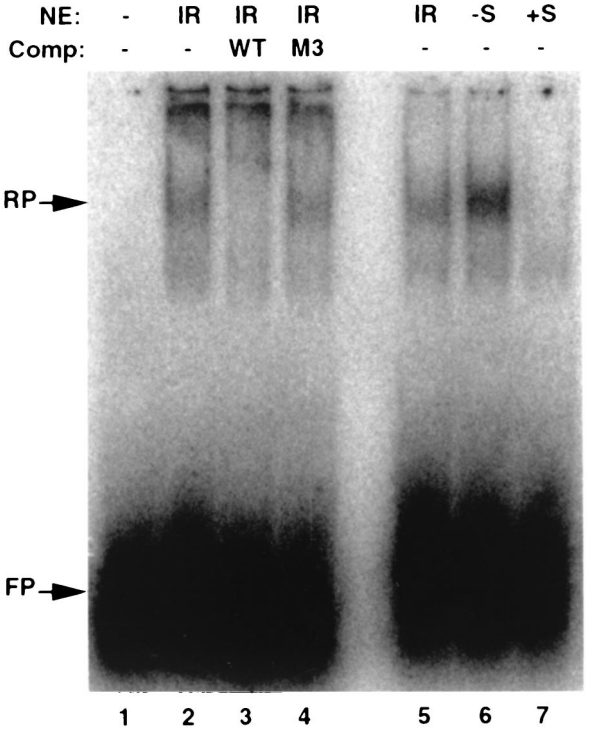

FIG. 7. Electrophoretic mobility shift assays. Nuclear extracts (NE) were prepared from WI-38 cells that had received 6 Gy of gamma irradiation 7 days before harvest (IR), that had been arrested in G_0/G_1 by confluence and serum starvation (−S), or that had been released from G_0/G_1 arrest by replating at a low density in medium with serum for 28 h (+S). Extracts were incubated with a 26-bp radiolabeled probe spanning the CDE and CHR region of the *CDK1* promoter. Unlabeled competitor (Comp) DNA identical to the probe (WT) or carrying mutation 3 (M3; Fig. 5) was added as indicated. Retarded probe (RP) and free probe (FP) were detected after nondenaturing polyacrylamide gel electrophoresis of binding reactions which contained 10 μg (lanes 2 to 4) or 5 μg (lanes 5 to 7) of NE.

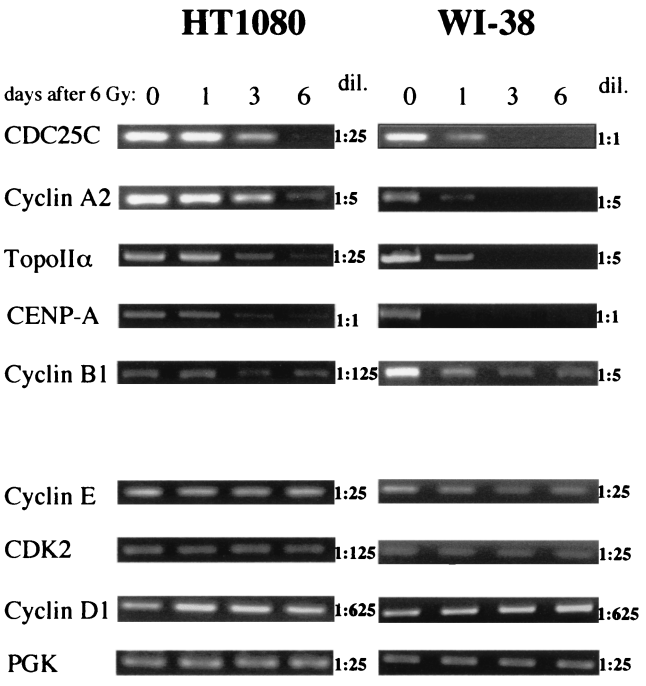

FIG. 8. RT-PCR assays for radiation-induced downregulation of transcripts of endogenous genes that do (upper panel) or do not (lower panel) show S/G_2-specific expression or have CHR and CDE promoter elements. HT1080 or WI-38 cells were gamma irradiated and harvested at the indicated times for RNA preparation. Reverse transcription products were used for PCR over a range of dilutions (dil.). Dilutions which generated visible but submaximal amounts of products at key times are shown. TopoIIα, topoisomerase IIα.

sion mechanism similar to that observed for *CDK1* is most likely.

DISCUSSION

In this paper, we have shown that transcription from the human *CDK1* promoter is repressed in response to DNA damage and that CDE and CHR elements are involved in this repression. We have shown further that the expression of other genes (those for cyclin A2, cyclin B1, CDC25C, CENP-A, and topoisomerase IIα) normally upregulated during S/G_2 and whose promoters include CDE and CHR elements is similarly silenced in response to DNA damage.

The CDE and CHR elements were discovered and characterized on the basis of their role in transcriptional derepression as cells progress from G_1 to S in the normal cell cycle (64, 65), but no role in mediating a response to DNA damage has previously been described. Of the various mutations we made in the *CDK1* promoter, mutations 3 and 5 which, respectively, completely change the CDE and CHR sequences, were the most effective at preventing downregulation. These results are in good agreement with those of Liu et al. (34), who showed that CDF-1 (CDE-CHR binding factor 1) interacts in a cooperative fashion with CDE and CHR and that it interacts with G residues in CDE (major groove of the DNA) and with A residues in CHR (minor groove). These G residues and A residues, respectively, were removed by mutations 3 and 5.

A previous study describing DNA damage-induced downregulation of CDK1 protein and mRNA in human primary fibroblasts (3) has recently been extended to show a similar downregulation of cyclin A, cyclin B, topoisomerase IIα, RAD51, and thymidine kinase (TK) (10). While transcriptional repression and the involvement of the CHR and CDE elements were not demonstrated in these reports, evidence that the response required functional p53 or p21 was presented. Our data are also consistent with a requirement for p53 in the response. Thus, p53 is known to be normal in HCT116 and HT1080 cell lines and can be presumed to be normal in primary WI-38 cells. In HeLa cells, the only cells in our study whose *CDK1* genes failed to be repressed in response to DNA damage, p53 activity is known to be blocked by interaction with the E6 gene product of human papillomavirus (49, 53).

We suggest that the relevant common feature of the coordinately downregulated genes is that they contain CDE and CHR elements in their promoters. As shown in Fig. 9A, all the genes that we or others (10) have shown to be downregulated by DNA damage (except the RAD51 gene, whose promoter sequence is not available in the database) have CDE and CHR elements shortly upstream of their transcriptional start sites.

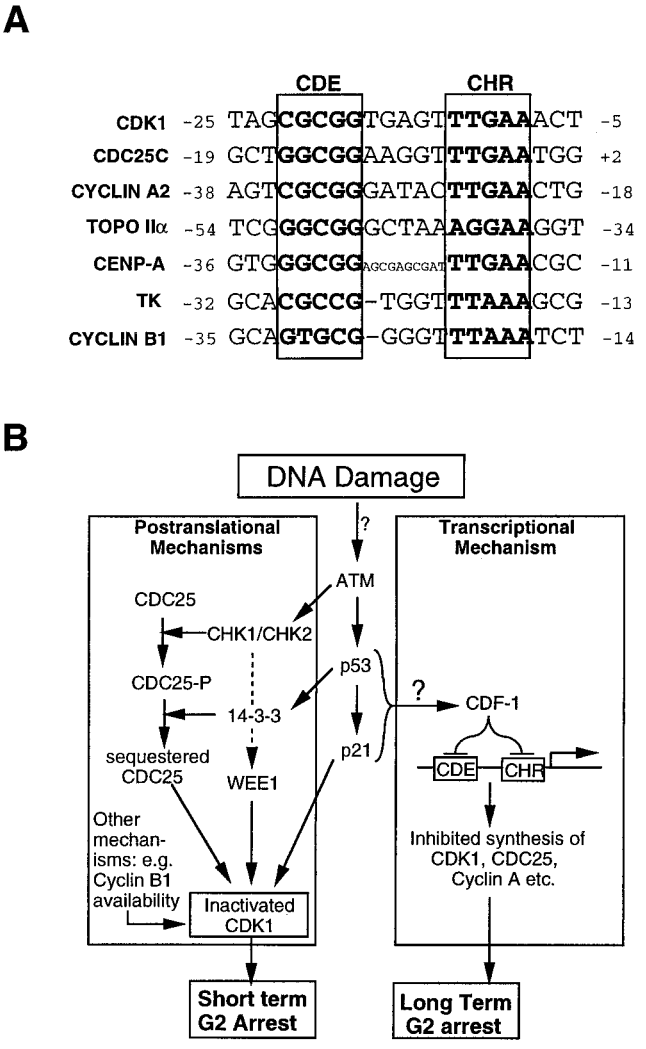

FIG. 9. (A) Nucleotide sequences for promoter regions of genes downregulated in response to gamma irradiation, showing characterized or putative CDE and CHR elements. Nucleotides are numbered relative to transcriptional start sites at position 1. Sources of sequence data were as follows: CDK1, CDC25C, and cyclin A2 (65), topoisomerase IIα (TOPO IIα) (24), CENP-A (50), TK (15), and cyclin B1 (30). (B) Model showing how the proposed transcriptional repression pathway might be integrated with previously proposed, largely postranslational DNA damage checkpoint pathways leading to G_2/M arrest. See the text for details.

These CDE and CHR elements have not previously been noted in the TK (15) and topoisomerase IIα (24) gene promoters and, in the latter case, the elements may well explain the observed p53-induced downregulation of the minimal promoter (48, 56). Similarly, reports of p53-dependent downregulation of cyclin A2 and cyclin B1 (4, 9) might be explained by the CDE and CHR elements in their gene promoters.

The results in this paper, combined with previous data (3, 10), support the existence of a p53- or p21-dependent pathway that senses DNA damage and culminates in transcriptional repression, most likely by CDF-1, of various S/G_2-associated genes. A link between p53 and CDF-1 is further supported by the recent observation that simian virus 40 large T antigen, which binds and inactivates p53, prevents CDF-1 from binding to CDE elements in WI-38 cells (63). Further components of this pathway and its role in DNA damage-induced G_2/M arrest are unknown at present, but it is interesting to speculate on these (Fig. 9). Thus, because the pathway is active at such late times after DNA damage and affects so many genes required for mitosis, we propose that its role may be to sustain or reinforce an otherwise transient G_2/M arrest established by other mechanisms, such as Chk1-mediated CDK1 phosphorylation. Alternatively, it may serve as a "fail-safe" mechanism for other G_2 DNA damage checkpoints. As for components of the pathway, it seems likely that ATM (5, 6, 36) and possibly DNA-dependent protein kinase (26, 60) act upstream of p53, while at present there are no obvious candidates for components acting downstream of p53 and p21. One possibility, consistent with the known action of p53 as a transcriptional activator, is that transcription of the CDF-1 gene is activated by p53.

It will be interesting to determine whether overexpression of a component of the pathway, especially CDF-1, leads to G_2 arrest. Artificial expression of p53 has been shown to induce (1) or reinforce (59) G_2 arrest in human cells, in the former case for up to 20 days. Although such arrest may involve CDF-1-mediated transcriptional repression, upregulation of 14-3-3σ (19) or some other action of the multifaceted p53 molecule could also be responsible.

The apparently slow kinetics of CDK1 transcriptional repression following DNA damage (Fig. 2 and 3), combined with the fact that inhibitory phosphorylation of CDK1 can be detected within minutes of DNA damage (31), seem to be consistent with a primary role for phosphorylation. However, because of the rapid accumulation of cells in G_2, when CDK1 is preferentially expressed, the observed kinetics of transcriptional repression may be misleading. Indeed, the opposing effect of G_2 accumulation may account for the net increase in CDK1 mRNA (Fig. 3), protein (Fig. 2), and kinase activity (Fig. 1) sometimes observed 1 day after DNA damage. Further work is therefore required to establish the exact timing of transcriptional downregulation relative to other key regulatory events, such as inhibitory phosphorylation of CDK1 and nuclear entry of cyclin B.

Of the five genes shown here to be downregulated by irradiation, the cyclin B1 gene was noticeably less responsive than the others (Fig. 8). This observation may reflect the fact that there are two different cyclin B1 transcripts, one that is cell cycle regulated (expressed predominantly in G_2/M) and another that is constitutively expressed (22); both would have been detected by our RT-PCR assay. However, data from other groups suggest that a mechanism distinct from the one that we have described may be responsible for the downregulation of cyclin B1 after irradiation. One report (30) suggests that the CDE element has a limited role in the correct cell cycle expression of cyclin B1, while another (23) describes p53-induced attenuation of the cyclin B1 promoter that was not accompanied by any decrease in CDK1 protein or mRNA levels. Thus, while transcriptional regulation of cyclin B1 can be an important part of the G_2 checkpoint (23, 29), it is possible that CDE- and CHR-independent mechanisms are involved.

In conclusion, we have shown that G_2 arrest after DNA damage is associated with the transcriptional repression of *CDK1* and the parallel downregulation of other several S/G_2-specific genes. The cell appears to have different ways of establishing G_2 arrest, including the control of CDK1 phosphorylation (27) and the nuclear localization of cyclin B (28). Transcriptional repression of *CDK1* and other genes required for mitosis represents another potential mechanism that may be particularly suitable for long-term G_2 arrest.

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C.B. and J.E.I. contributed equally to this work.

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