Transcriptional Regulation of Mitotic Checkpoint Gene MAD1 by p53

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p53 regulates a number of genes through transcriptional activation and repression. p53-dependent mitotic checkpoint has been described, but the underlying mechanism is still obscure. Here we examined the effect of p53 on the expression of a human mitotic checkpoint protein, Mitosis Arrest Deficiency 1 (MAD1), in cultured human cells. The expression of MAD1 was reduced when the cells were overexpressing exogenously introduced wild-type p53. The same reduction was also observed when the cells were treated with anticancer agents 5-fluorouracil and cisplatin or were irradiated with UV. Consistently, MAD1 promoter activity diminished in a dose-dependent manner when induced by p53, indicating that p53 repressed MAD1 at a transcriptional level. Intriguingly, several tumor hot spot mutations in p53 (V143A, R175H, R248W, and R273H) did not abolish the ability of p53 to repress MAD1 expression. By serial truncation of the MAD1 promoter, we confirmed that p53-responsive element to a 38-bp region that represents a novel sequence distinct from the known p53 consensus binding site. Trichostatin A, a histone deacetylase inhibitor, relieved the p53 transrepression activity on MAD1. Chromatin immunoprecipitation assay revealed that p53, histone deacetylase 1, and co-repressor mSin3a associated with the MAD1 promoter in vivo. Taken together, our findings suggest a regulatory mechanism for the mitotic checkpoint in which MAD1 is inhibited by p53.

The p53 tumor suppressor gene takes part in cell cycle control, DNA damage repair, and apoptosis (1–3). Its importance is underscored by the fact that p53 gene is frequently mutated in more than 50% of all human tumors. p53 acts as a transcription factor regulating numerous downstream target genes. It can transactivate genes involved in cell cycle progression and apoptosis, such as p21WAF1, MDM2, BCL2, and BAX (4–6). Transcriptional activation requires p53 binding to a consensus sequence TATA-binding protein (15) and Sp1 (16).

The mitotic checkpoint prevents the onset of anaphase by blocking the activation of anaphase-promoting complex (APC) until all sister chromatids have aligned properly to the spindle and attached in a bipolar manner (17, 18). Several checkpoint components were initially identified in budding yeast, including MAD1, MAD2, MAD3, BUB1, BUB2, BUB3, and MPS1. Vertebrate homologs of MAD1, MAD2, BUB1, and BUB3 localize to kinetochores prior to chromosome alignment on the metaphase plate (19, 20). Loss of mitotic checkpoint function is associated with chromosomal instability in cancer cells (21–25). MAD2 appears to be the critical component in the mitotic checkpoint pathway. Unattached kinetochores recruit MAD2 and other checkpoint proteins, thereby activating the checkpoint (18). MAD2 is able to bind Cdc20, an activator of APC, and prevents Cdc20 from association with APC, resulting in APC inhibition (17, 18). The checkpoint ends with bipolar attachment at metaphase. Several checkpoint proteins including MAD2 move away from kinetochores, releasing Cdc20 to activate APC (26, 27). Two main substrates for the APC are mitotic cyclins (A and B) and securin, and their destruction is required for the exit from mitosis and sister chromatid segregation, respectively (28–31). During interphase, MAD1 and MAD2 localize to the nuclear pores (32–34). MAD1 and Cdc20 can bind MAD2, but these two bindings are exclusive. They share a 10-residue MAD2-binding motif. It has been suggested that MAD1 acts as a positive regulator and a competitive inhibitor of the MAD2-Cdc20 complex (35–38). The structure and function of MAD2 are relatively well understood, but little information about MAD1 is known thus far. In a serial analysis of gene expression (SAGE) in human colorectal cancer cells expressing p53 versus the p53 non-expressing parental cells, MAD1 has been identified as one of the genes regulated by p53 (39). Here we attempted to investigate in depth the role of p53 in MAD1 expression. We show that wild-type and mutant p53 transcriptionally repress MAD1 in human primary cells and several cell

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lines including HepG2 and Hep3B. Moreover, we mapped the p53-responsive element in the MAD1 promoter. p53 repression activity on MAD1 is decreased upon addition of histone deacetylase inhibitor, trichostatin A. Significantly, p53 and mSin3a as well as HDAC bind to the MAD1 promoter in vivo, consistent with a recently proposed model in which these three proteins form a repression complex targeting the p53-repressive genes. Notably, the binding of mSin3a and HDAC to the MAD1 promoter is p53-dependent.

EXPERIMENTAL PROCEDURES

Plasmids—The MAD1 promoter containing 480 bp upstream of the exon 1 of MAD1 (designated as −428) was amplified from human genomic DNA (Novagen) using the primers 5′-AATCGCAGCTCTTGCTGCAAGGACCTGG-3′ (forward) and 5′-GCTTCTAGAATCTATCCGTCGCGGCTGCGAG-3′ (reverse). Four truncated the MAD1 promoters (−349, −311, −231, and −102) were amplified by PCR using −428 template, with the following forward primers: for −349, 5′-AATCTGCAGCTCAGATTGTCAAGTGA-3′; for −311, 5′-AATCTGCAGGGAGAAAGTTAACAAGGTGCAATG-3′; for −231, 5′-AATCTGCAGCCTTGATTGAAAGCCCCTCCTGGCAGA-3′; and for −102, 5′-AATCTGCAGGAGATCGACCGGTGCGGACTC-3′; and with the same reverse primer as shown above for −428. PsI and XbaI restriction sites (underlined) were generated in forward and reverse primers, respectively; and the amplified promoter fragments were cloned into CAT reporter plasmid pCAT-Basic lacking eukaryotic promoter and enhancer sequences (Promega). All constructs were confirmed by DNA sequencing.

p53 expression vectors with wild-type (wt), pCMVp53, and with mutations at amino acids residues 143 (Val for His), 248 (Ala for Leu), 311, 349, and 428 as targets were expressed in the colorectal cancer cell line DLD-1 (39). To understand further the role of p53 in regulating MAD1, we transiently transfected the human hepatoblastoma cell line HepG2 with a CMV promoter-driven p53-expression plasmid pCMVp53. The expression of p53 and endogenous MAD1 was detected by Western blotting. As shown in Fig. 1A, the level of MAD1 protein decreased significantly when cells were transfected with a low dose of p53-expressing plasmid (0.1 μg, lane 2) compared with the mock-transfected control cells (lane 1). Notably, the MAD1 level decreased in a dose-dependent manner. When a relatively large quantity of p53 (12.5 μg) was added, endogenous MAD1 protein was minimally detected (lane 4).

RESULTS

Down-regulation of MAD1 Protein Expression by Wild-type p53—A genome-wide SAGE analysis revealed that human mitotic checkpoint gene MAD1 is induced by p53 in the colorectal cancer cell line DLD-1 (39). To understand further the role of p53 in regulating MAD1, we transiently transfected the human hepatoblastoma cell line HepG2 with a CMV promoter-driven p53-expression plasmid pCMVp53. The expression of p53 and endogenous MAD1 was detected by Western blotting. As shown in Fig. 1A, the level of MAD1 protein decreased significantly when cells were transfected with a low dose of p53-expressing plasmid (0.1 μg, lane 2) compared with the mock-transfected control cells (lane 1). Notably, the MAD1 level decreased in a dose-dependent manner. When a relatively large quantity of p53 (12.5 μg) was added, endogenous MAD1 protein was minimally detected (lane 4).

In control experiments, we first queried whether the expression vector alone without p53 could lead to the decrease of MAD1 expression. p53 was cloned previously (22).
immunoblot (lane 3). These results indicate that p53 down-regulates the expression of MAD1 protein in a dose-dependent manner. The down-regulation of MAD1 was also observed in two p53-null cell lines, Hep3B (hepatocellular carcinoma) and PC-3 (metastatic prostate cancer), as well as in IMR-90 cells (normal embryonic lung fibroblast) with wild-type p53, when these cells were transfected with p53 (Fig. 1D). Hence, this repression was unlikely cell type-specific.

To characterize further the p53 effect at the single cell level, we performed immunofluorescence studies using the aforementioned anti-MAD1 antiserum 181d. As a first step, we verified the specificity of the 181d antiserum in immunofluorescence staining. We transiently transfected HeLa cells with MAD1 expression plasmid and stained the cells with 181d antiserum (Fig. 2A, panel 1), and with a previously characterized antiserum 105 highly specific for human MAD1 (22). Both antibodies specifically reacted with both the endogenous and the overexpressed MAD1 protein (Fig. 2A, compare cells with arrows to cells without arrows). This nuclear localization pattern with some concentration in the nuclear membrane was typical to cells expressing MAD1 protein (Fig. 2A, panel 1). This induction of p53 by chemotherapeutics considerably repressed the expression of MAD1 (panel 4). Thus, p53 repressed MAD1 expression specifically.

Repression of MAD1 Expression upon Treatment with Chemotherapeutics in HepG2 Cells—Having observed that introduction of exogenous p53 into cells could repress the expression of MAD1, we were interested in examining whether such repression could be sustained in a physiological environment with an elevated amount of endogenous p53. Treatment of cells with chemotherapeutics such as DNA-damaging agents 5-fluorouracil (5-FU) and cisplatin has been documented to induce expression of endogenous p53. These agents have been widely employed in the studies of cell cycle regulation and apoptosis (51, 52). Two human hepatocarcinoma cell lines HepG2 and Hep3B were treated with increasing concentrations of 5-FU and cisplatin, followed by analysis for the expression of MAD1 and p53 by immunoblotting, using β-actin as a reference to verify for the equivalent loading of samples.

Repression of MAD1 expression by p53 in human cells. A, decrease of MAD1 protein expression in the presence of p53. HepG2 cells were transfected with 0.1, 0.5, and 2.5 μg of wild-type p53-expression plasmids pCMVp53, respectively (lanes 2–4). The total amount of transfected DNA in each sample was held constant using pUC19 plasmid DNA to compensate. Equal protein concentration was loaded in each lane. The blot was incubated with polyclonal anti-MAD1 (181d; top) or with monoclonal anti-p53 antibody (DO-1; bottom). Cells not transfected with the p53-expression plasmid provide the background signal (lane 1). B, the effect of pCMV empty vector on MAD1 protein expression. p53 was cloned downstream of a CMV promoter in the pCMV vector (50). HepG2 cells were transfected with 0.1, 0.5, and 2.5 μg of pCMV empty vector, respectively (lanes 2–4). The blot was incubated with MAD1 181d antibody (top) or with anti-β-actin antibody (AC-40, bottom) to verify equal sample loading. C, specificity of anti-MAD1 antibody (Ab). 10, 25, and 25 μg (lanes 1–3) of the untransfected control cell lysates (A, lane 1) were applied onto SDS-PAGE and analyzed by Western blotting. The blot was incubated with polyclonal 181d antibody (lanes 1 and 2) or with 181d blocked with 5 ng of MAD1 peptide (lane 3, blocked 181d). D, the effect of p53 on MAD1 protein expression in fibroblasts and in cancer cell lines. p53-deficient Hep3B and PC-3 cells, and IMR-90 cells with wild-type p53 were transfected with 1, 0.5, and 0.5 μg of pCMVp53 (+), respectively, or with the same amount of pCMV empty vector (–). Cells were lysed, and the same quantities of samples were analyzed for the protein expression of MAD1 and p53 by immunoblotting, using β-actin as a reference to verify for the equivalent loading of samples.

Next we assessed the influence of p53 on MAD1 expression by transfecting HeLa cells with p53. We observed that the overexpression of p53 (Fig. 2B, panel 1) correlated with a dramatic reduction in the steady-state amount of MAD1 protein in HeLa cells (panel 2, cell with arrow), whereas an appreciable amount of MAD1 was retained in the nucleus with a low basal concentration of endogenous p53 (cell with asterisk). As a negative control, the overexpression of the Gal4 DNA-binding domain (Gal4BD; Fig. 2B, panel 3) had no effect on the expression of MAD1 (panel 4). Thus, p53 repressed MAD1 expression specifically.

Having observed that introduction of exogenous p53 into cells could repress the expression of MAD1, we were interested in examining whether such repression could be sustained in a physiological environment with an elevated amount of endogenous p53. Treatment of cells with chemotherapeutics such as DNA-damaging agents 5-fluorouracil (5-FU) and cisplatin has been documented to induce the expression of endogenous p53. These agents have been widely employed in the studies of cell cycle regulation and apoptosis (51, 52). Two human hepatocarcinoma cell lines HepG2 and Hep3B were treated with increasing concentrations of 5-FU and cisplatin, followed by analysis for the expression of MAD1 and p53 by Western blotting. Both 5-FU and cisplatin effectively induced endogenous p53 expression over the basal level in p53-expressing HepG2 cells (Fig. 3A, left panel, lanes I–5). The induction of p53 by chemotherapeutics considerably repressed the expression of MAD1 in the cells. In both drug treatments, the down-regulation of MAD1 again followed a dose-dependent manner, consistent with the pattern observed
in the cells transfected with p53-expression plasmids (Fig. 1). Here, p53-deficient Hep3B cells served as a negative control because a major portion of the p53 gene in Hep3B cells was found to be deleted, accompanied by the absence of p53 RNA transcripts and protein (42, 43). Thus, no p53 protein was detected in Hep3B cells even in the presence of p53-inducing drugs (Fig. 3A, right panel, lanes 1–5). As a positive control, Hep3B cells were transfected with pCMVp53, and the p53 protein was detected in Western blotting (Fig. 3A, right panel, lanes 6, 181d, and 181d + pep.). Arrows highlight transfected cells. Bar, 20 μm. B, p53 repression of MAD1 expression. HeLa cells were transfected with pCMVp53 (panels 1 and 2) or plasmid expressing the Gal4 DNA-binding domain as negative control (panels 3 and 4). Cells were fixed and co-stained for p53 (panel 1) or Gal4 (panel 3) and MAD1 (panels 2 and 4). Arrows indicate transfected cells, and asterisks highlight untransfected cells. The same fields are shown in panels 1–4. Bar, 20 μm.

Next we sought to examine whether these results were applicable to other carcinoma cells and normal primary cells. HeLa (cervical carcinoma), CNE2 (nasopharyngeal carcinoma), MCF-7 (breast carcinoma), LoVo (colon carcinoma), and IMR-90 (normal lung fibroblast) cells harboring wild-type p53 displayed a reduction of protein expression level of MAD1 when they were treated with cisplatin. PC-3 (prostate carcinoma) and SK-OV-3 (ovarian carcinoma) cells, like Hep3B, were null for p53. Thus, the addition of p53-inducing drugs had no effect on the alteration of MAD1 expression (Fig. 3B). The cisplatin-induced down-regulation of MAD1 expression is p53-dependent, and this finding agreed with the decrease of MAD1 observed in the cells transfected with exogenous p53 (Fig. 1). Although a previous report (39) describes an induction of MAD1 in the DLD-1 colon cancer cell line upon infection with adenovirus encoding p53, we did not detect any increase in the levels of MAD1 upon elevated expression of p53 using two distinct approaches in the cell lines we have studied.

The data shown above manifest the fact that p53 represses MAD1 expression in both tumor and non-tumor cells. There are two possible mechanisms to explain the observation: p53 influenced either the protein level (i.e., protein degradation) or the transcription of MAD1. A number of cell cycle regulatory proteins such as cyclins, securin, and p53 were degraded by ubiquitination. Proteasome inhibitors MG115 (25 μM) and PSI (30 μM) were added to cisplatin-treated HepG2, IMR-90, and MCF-7 cells for 5 h before harvest (lanes 3 and 4, respectively). Cells were lysed, and protein expression levels were analyzed by Western blotting using MAD1-, p53-, and β-actin-specific antibodies.

**Fig. 2. Repression of MAD1 expression by p53 in HeLa cells.** A, verification of antibody specificity. HeLa cells were transfected with MAD1 expression plasmid (22), fixed 42 h after transfection and stained with MAD1 antibodies 181d (panel 1), 105 (panel 2), and 181d pre-incubated with an excess amount (5 μg) of the immunizing peptide (panel 3, 181d + pep.). Arrows highlight transfected cells. Bar, 20 μm. B, p53 repression of MAD1 expression. HeLa cells were transfected with pCMVp53 (panels 1 and 2) or plasmid expressing the Gal4 DNA-binding domain as negative control (panels 3 and 4). Cells were fixed and co-stained for p53 (panel 1) or Gal4 (panel 3) and MAD1 (panels 2 and 4). Arrows indicate transfected cells, and asterisks highlight untransfected cells. The same fields are shown in panels 1–4. Bar, 20 μm.

**Fig. 3. Repression of MAD1 expression by endogenous p53 induced in response to chemotherapeutics.** A, dose-dependent induction of p53 and repression of MAD1 upon treatment with chemotherapeutics in p53-positive HepG2 cells but not in p53-negative Hep3B cells. HepG2 and Hep3B cells were treated with 1 and 10 μg/ml of 5-fluorouracil (5-FU) (lanes 2 and 3, respectively) or with 1 and 10 μg/ml of cisplatin (CIS) (lanes 4 and 5, respectively), or not treated with any drugs (lane 1). Hep3B cells transfected with 3 μg of pCMVp53 serve as a positive control (C, lane 6). The blot was incubated with anti-MAD1 (181d; top) or anti-p53 (DO-1; bottom) antibody to reveal the respective protein expression levels. B, cisplatin induces p53 and reduces MAD1 protein levels in p53-positive cell lines but not in p53-negative cell lines. HeLa, CNE2, MCF-7, LoVo, IMR-90, PC-3, and SK-OV-3 cells were treated with 10 μg/ml of cisplatin (CIS) (lanes 2 and 3, respectively) or with 1 and 10 μg/ml of cisplatin (CIS) (lanes 4 and 5, respectively), or not treated with any drugs (lane 1). Hep3B cells transfected with 3 μg of pCMVp53 serve as a positive control (C, lane 6). The blot was incubated with anti-MAD1 (181d; top) or anti-p53 (DO-1; bottom) antibody to reveal the respective protein expression levels. B, cisplatin induces p53 and reduces MAD1 protein levels in p53-positive cell lines but not in p53-negative cell lines. HeLa, CNE2, MCF-7, LoVo, IMR-90, PC-3, and SK-OV-3 cells were treated with 10 μg/ml of cisplatin (CIS) (lanes 2 and 3, respectively) or with 1 and 10 μg/ml of cisplatin (CIS) (lanes 4 and 5, respectively), or not treated with any drugs (lane 1). Hep3B cells transfected with 3 μg of pCMVp53 serve as a positive control (C, lane 6). The blot was incubated with anti-MAD1 (181d; top) or anti-p53 (DO-1; bottom) antibody to reveal the respective protein expression levels. B, cisplatin induces p53 and reduces MAD1 protein levels in p53-positive cell lines but not in p53-negative cell lines. HeLa, CNE2, MCF-7, LoVo, IMR-90, PC-3, and SK-OV-3 cells were treated with 10 μg/ml of cisplatin (CIS) (lanes 2 and 3, respectively) or with 1 and 10 μg/ml of cisplatin (CIS) (lanes 4 and 5, respectively), or not treated with any drugs (lane 1). Hep3B cells transfected with 3 μg of pCMVp53 serve as a positive control (C, lane 6). The blot was incubated with anti-MAD1 (181d; top) or anti-p53 (DO-1; bottom) antibody to reveal the respective protein expression levels.
uitin-mediated proteolysis (6, 28–31). On the other hand, p53 can in turn regulate the synthesis of ubiquitin protein ligases such as Mdm2 (6). To investigate whether p53 has an impact on the proteasome-dependent degradation of MAD1 protein, we evaluated the effect of proteasome inhibitors on the levels of MAD1 in cisplatin-treated cells. Cells harboring wild-type p53, including HepG2, IMR-90, and MCF-7, were treated with cisplatin to induce endogenous expression of p53 and then with proteasome inhibitors MG115 and PSI for 5 h. If p53 regulates MAD1 via the ubiquitin-proteasome pathway, the impediment of proteasome activity by the inhibitors should relieve the repressive effect by cisplatin, resulting in a rise in MAD1 protein levels comparable with the levels in cells without cisplatin treatment (Fig. 3C, lane 1). When cells were treated with cisplatin, p53 was induced, and MAD1 protein level was declined, as expected (lane 2). However, we did not observe any increase of MAD1 protein levels upon addition of MG115 or PSI (lanes 3 and 4). Rather, the levels remained repressed in all three cells we analyzed. This assay vigorously indicated that repression of MAD1 protein expression by p53 was unlikely mediated through the ubiquitin-proteasome degradation pathway.

Expression of MAD1 Is Regulated by p53 at Transcriptional Level—After eliminating the possibility of p53-mediated ubiquitination of MAD1, we asked whether the repression occurred at the stage of transcription. We amplified a region with 480 bp upstream of the exon 1 of human MAD1 by PCR (Fig. 4A) and inserted it into a CAT reporter (pCAT-Basic vector), which lacked eukaryotic promoter and enhancer sequences. The resulting plasmid is named p428CAT. This 480-bp sequence did not contain p53 consensus binding site comprising two copies of 5’-RRRC(A/T)/T(A)GGG-3’ separated by 0–13 bp (7). A putative TATA box was found in the sequence at −171/−13 (Fig. 4A, underlined) upstream of the transcription start site (+1, arrow). HepG2 and Hep3B cells were transiently transfected either with p428CAT or with the parental pCAT-Basic vector and analyzed for CAT activities (Fig. 4B). Because the pCAT-Basic vector did not contain any promoter or enhancer sequences, detectable CAT activity indicated that the 480-bp sequence possessed promoter activity. Indeed, the CAT assay demonstrated that the 480-bp sequence acquired a considerable promoter activity when compared with the control (Fig. 4B, upper panel, compare lane 2 with 1). By employing this CAT reporter construct driven by the MAD1 promoter, we then investigated the effect of p53 using the CAT assay. In addition to chemotherapeutics, UV irradiation is another well known stimulus inducing p53 expression in cells (53, 54). HepG2 and Hep3B cells both transfected with p428CAT plasmid were exposed to UV at 50 J/m² and harvested 24 h after irradiation. Upon UV irradiation, an abundant increase of endogenous p53 over the basal expression level was detected in HepG2 cells but not in p53-null Hep3B cells by Western blotting (Fig. 4B, lower panel, compare lane 3 with lanes 1 and 2). Correspondingly, the MAD1 promoter activity diminished in HepG2 cells as a result of the induction of p53 by UV irradiation, whereas the activity remained unchanged in Hep3B cells (upper panel) even at 100 J/m² of UV (data not shown). In line with the previous cisplatin treatment assay, UV-induced decline of MAD1 promoter activity is also p53-dependent, and these data further illustrated that repression of MAD1 occurred at a transcriptional step. To ascertain the effect of UV irradiation on MAD1 promoter activity, we had both HepG2 and Hep3B cells exposed to UV irradiation and analyzed p53 and MAD1 proteins (Fig. 4C). Consistent with the CAT assay, Western blot analysis showed that UV-induced p53 down-regulated MAD1 protein expression through the lowering of the MAD1 promoter activity.

To confirm further the effect of p53 on the MAD1 promoter, we used p53-null Hep3B cells. Because Hep3B cells are deficient in p53, we transiently co-transfected pCMVp53 and p428CAT into the cells, instead of using p53-inducing agents such as anticancer drugs or UV, to provide a source of p53. Cells transfected with p428CAT exhibited robust basal CAT activity (Fig. 4D, lane 2). Nevertheless, the promoter activity diminished in the presence of progressively increasing amounts of p53 plasmids (lanes 3–5). When 12.5 μg of pCMVp53 was added (lane 5), the activity reduced down to only 5% of that in the absence of p53 (lane 2). Because the total DNA amounts were kept constant by compensating with an irrelevant plasmid mid pUC19 in all dose-dependent assays, it indicated that the effect of p53 on MAD1 promoter activity was specific and was not due to an increase in the total amount of DNA transfected. The same CAT assay was also conducted in p53-expressing HepG2 and HeLa cell lines, and the results agreed with those obtained in Hep3B cells (data not shown). These results clearly demonstrated that p53 transcriptionally repressed the expression of MAD1 in a dose-dependent manner, and this effect seemed not to be cell type-specific.

Transcriptional Repression of MAD1 by p53 Mutants—Because p53 is the most frequently mutated gene identified in human cancers (>50% of all cancers) and mutation of p53 generally resulted in the inactivation of the tumor suppressor function (1), it is of interest to study the influence of p53 mutants on MAD1 promoter activity. Here we looked at four p53 mutants that contain changes at codon 143 (Val to Ala), 175 (Arg to His), 248 (Arg to Trp), and 273 (Arg to His), respectively. All these four p53 mutants represent tumor “hot spots” and have been found to be defective in binding to p53 consensus binding sequence (7, 40). We used p53-null Hep3B cells, rather than p53-positive cell lines such as HepG2 or HeLa, to avoid any confounding effects from the endogenous p53. Hep3B cells were co-transfected with either wild-type or mutant p53 with indicated amounts and an equal amount (5 μg) of p428CAT. As expected, wild-type p53 repressed MAD1 promoter activity (Fig. 5, lane 2 and 3). Surprisingly, all four p53 mutants also exhibited the ability to transcriptionally repress MAD1 with various extents. Markedly, the down-regulation of MAD1 promoter activity by these mutants acted in a dose-dependent manner as the wild-type p53, with the lowest MAD1 promoter activity at the highest concentration of p53 (lanes 4–15).

Identification of a p53-responsive Element in the MAD1 Promoter—Knowing that the transactivation by p53 is mediated through direct binding of the protein to its binding consensus on target genes (7, 55, 56), we inspected the MAD1 promoter, but there was no match for the p53-binding site. Recently, an analysis of p53-binding elements showed that the previously defined p53 binding consensus comprising two copies of the sequence RRRC(A/T) arranged in a head-to-head fashion, with 0–13 nucleotides apart (7), allowed p53 activation of the target genes, whereas a head-to-tail arrangement shifted the activity of p53 from activation to repression (57). Nevertheless, none of these sequence arrangements were found in the MAD1 promoter. The absence of p53-binding consensus prompted us to search for the p53-responsive region in the MAD1 promoter. Serial truncation of the MAD1 promoter was constructed by PCR amplification, and these fragments were cloned upstream of the CAT gene in pCAT-Basic vector, as represented in the schematic diagram (Fig. 6A). Hep3B cells were transfected with these truncated constructs alone or together with wild-type or p53 mutant R175H and assayed for CAT activities (Fig. 6B). Even though 326 bp were deleted from the 5’ of 480-bp MAD1 promoter (i.e. p102CAT), robust basal promoter activity
**FIG. 4. Definition of MAD1 promoter and transcriptional repression of MAD1 promoter by p53 in HepG2 and Hep3B cells.**

_A_, DNA sequence of human MAD1 promoter. The 480-nucleotide sequence shown is located immediately upstream of the exon 1 of human MAD1 gene. The putative TATA sequence (−17/−13) is _underlined_; the transcription start site predicted is indicated (arrow) and is numbered as +1, and the
p53 Represses MADI Expression

Fig. 5. Transcriptional repression of MADI by p53 mutants in Hep3B. Hep3B cells were transfected with p428CAT in the absence (lane 1) or presence (lanes 2–15) of various p53-expression plasmids. p53 wild-type (wt, lanes 2 and 3), V143A (lanes 4–6), R175H (lanes 7–9), R248W (lanes 10–12), and R273H (lanes 13–15) mutant plasmids with indicated amounts (µg) were added to cells, respectively. Cells were lysed, and lysates of equal concentration were assayed for their CAT activity (top) and were analyzed by Western blotting using anti-p53 antibody (DO-1; bottom). The result shown is representative of three independent experiments.

was still retained (lane 12). This suggested that the minimal promoter region may reside within the region of 154 bp between +52 and −102. Like wild-type p53, R175H mutant displayed repression activity on the 480-bp MADI promoter, albeit weaker activity than the wild-type by about 4-fold (compare lane 5 with lane 1). After all, p53 was not able to repress the promoter with 117 nucleotides deleted from the 5' (i.e. p311CAT) or with further truncations (i.e. p231CAT and p102CAT). The restoration of promoter activities in p428CAT versus p311CAT in the presence of wild-type and mutant p53 consistently suggested that the p53-responsive element was located in a region of 117 bp between −311 and −428 of MADI promoter.

To finely map the p53-responsive region, we generated another deletion between −311 and −428, with 79 nucleotides removed from the 5' of the MADI promoter (i.e. p349CAT, Fig. 7A). The promoter activity of p349CAT was repressed in the presence of wild-type p53, resembling that of the p428CAT (Fig. 7B). The repression by p53, however, was abolished when the promoter was truncated down to position −311, where an appreciable increase of promoter activity was observed. The equivalent amounts of p53 protein expressed in all p53-transfected samples as shown by Western blotting (Fig. 7B, lower panel, lanes 1–3) eliminated the possibility that the rise of promoter activity was due to the decline of p53 expression. The promoter truncation experiments thus distinctly delineated the element responsible for p53 repression within a region of 38 bp between −311 and −349 of MADI promoter (Fig. 4A, dashed line).

Moderation of p53-mediated Repression of MADI by Trichostatin A—It is generally thought that transcriptional repressors are often associated with histone deacetylases (HDACs) because deacetylases have been shown to be involved in several gene repression systems (58). The histone deacetylase activity in particular is required for the HDAC1-mediated repression of target genes (59). First, we addressed whether histone deacetylase activity is required for the repression of MADI by p53. Hep3B cells were transiently co-transfected with p428CAT and wild-type p53, in the absence or presence of various concentrations of trichostatin A (TSA). TSA is a potent and specific inhibitor of HDACs in vitro and in vivo (60). Although wild-type p53 repressed MADI promoter activity (Fig. 8, lane 7), addition of TSA seemed to alleviate the repression (lanes 8–11). The MADI promoter activity increased −3-fold when 2 µM of TSA was added to the culture medium (compare lane 7 with lane 8). However, the promoter activity did not increase further even at higher concentrations up to 5 µM TSA (lane 11). Although the repression could not be completely abrogated by TSA, the demeption of MADI promoter activity by TSA in the presence of p53 is prominent. Western blot analyses showed that the amounts of p53 were relatively constant in the absence and presence of TSA, ruling out the possibility that the increase of promoter activity was due to the decline of p53 protein expression. The experiment was repeated using p53 mutant R175H instead of wild-type p53, and de-repression of MADI promoter activity was also observed in the presence of TSA (data not shown). A previous study (61) has demonstrated that TSA alone was able to induce the bcl-2 minimal promoter activity. To determine whether TSA exhibited the same effect on MADI promoter, we assayed the CAT activity in cells transfected only with p428CAT without pCMVp53 in the presence of TSA. There was no apparent increase in MADI promoter activity when cells were treated with TSA (compare lane 1 with lanes 2–6). These data illustrate the ability of TSA to revert the p53-dependent repression, suggesting that histone deacetylase activity is implicated in the repression mechanism.

p53, HDAC1, and mSin3a Are Associated with the MADI Promoter in Vivo—HDAC1 has been found to interact with a transcriptional co-repressor mSin3a in a multiprotein complex which represses transcription of many genes (62). Recent reports (10, 61) revealed that p53 forms a complex with mSin3a and HDAC1 in vivo, and this interaction is critical for p53-mediated transcriptional repression of at least some target genes. The TSA effect described above strongly suggests that p53 might repress the transcription of MADI through HDACs. Therefore, we explored the interaction of individual protein in the HDAC1 repression complex with endogenous MADI promoter by chromatin immunoprecipitation (ChIP) using antibodies specific for p53, HDAC1, and mSin3a, as well as using pre-immune serum (IgG) as a negative control. p53-null Hep3B cells were transfected with empty vector or pCMVp53, followed by formaldehyde treatment. The cross-linked chromatin from these two samples of cells was then immunoprecipitated with individual antibodies. The chromatin DNA was eluted after reversal of cross-links and treatment by proteinase K and RNase A. The cross-linked samples without adding antibody...
was reserved as total input. The eluted DNA was PCR-amplified using primers specific for the MAD1 promoter. As controls, p428CAT reporter was used as a template in PCR to reveal the amplified signal, and water was also used as a template to show the background. As shown in Fig. 9, all three antibodies specific for p53, HDAC1, and mSin3a readily immunoprecipitated endogenous MAD1 promoter (lanes 10–12) in p53-transfected cells. HDAC1 and mSin3a bound to the MAD1 promoter in the absence of p53 (lanes 5–7), suggesting that the recruitment of HDAC1 and mSin3a to the MAD1 promoter in vivo is p53-dependent.

**DISCUSSION**

As a “guardian of the genome,” tumor suppressor p53 protein is activated in response to a variety of stimuli and stresses such as DNA damage, hypoxia, microtubule disruption, and activated oncogenes (2). Activated p53 is primarily involved in G1/S or G2/M arrest in the cell cycle. One well characterized function of p53 is the transactivation of its downstream target genes via specific DNA sequence binding (7). p53 also exhibits transcriptional repression activity on a diverse set of genes. Here we present another p53-regulated target, a mitotic checkpoint protein, MAD1. In the current working model for mitotic checkpoint control, MAD1 recruits MAD2 to the unattached kinetochore during prometaphase, thereby eliciting the checkpoint. After dissociation from MAD1, MAD2 no longer localizes to kinetochore and dissociates from CDC20, thus inducing APC activation and anaphase onset (17). Although
MAD1 can be modified by phosphorylation in vitro by MPS1 and BUB1 (18), regulation of MAD1 remains elusive. In this study, we demonstrate that MAD1 is repressed by p53 at a transcriptional level.

Transcriptional Repression of MAD1 Expression by p53—p53-induced transcriptional repression of genes involved in G2/M transition include cyclin B1 (63), CDC2 (14), and CDC25C (64), and recent studies (65, 66) have shown that the anti-apoptotic surviving gene, survivin, is another p53 repressible gene. Survivin is maximally expressed in the G2/M phase of 1/H9262 cells, implicating p53 in mitotic checkpoint (72). Therefore, it is not surprising that p53 is also involved in regulation of mitotic checkpoint genes.

In vitro, MAD1 can be manipulated by the expression of wild-type p53 (wt-p53) and gain-of-function p53 mutant (p53G281D) (74, 75). It is not fully understood whether and how the p53 241F mutant might interfere with the regulation of MAD1 by wild-type p53. During the preparation of this manuscript, an independent study on p53 regulation of MAD1 in DLD-1 was again observed under these conditions, consistent with the results obtained by transfecting cells with p53-expressing vectors (39) or in the presence of 1 μg of wt-p53 plasmid pCMVp53 (lanes 7–11). TSA of various concentrations as indicated was incubated during transfection of the cells (lanes 2–6 and 8–11). Cells were harvested 16 h after transfection and lysed, followed by CAT assay (top). Cells without TSA treatment serve as control (lanes 1 and 7). Western blotting using anti-p53 (DO-1), anti-HDAC1 (H-11), and anti-mSin3a (G-11) antibodies (bottom) was performed.

MAD1, previously characterized to be a Tax-binding protein (also known as TXBP 181) (22), has been found using the SAGE technique to be a gene (PIG9) up-regulated in response to p53 expression in colorectal cancer cell DLD-1 (39). In light of this, we intended to further characterize the regulation of MAD1 by p53 in the present study. Surprisingly, our results reproducibly indicate that MAD1 is repressed by p53. Transient transfection of exogenous p53 into HepG2, IMR-90, Hep3B, PC-3, and HeLa cells yielded a decline of MAD1 expression (Fig. 1 and Fig. 2). To eliminate the possibility of artifacts created by transfecting endogenous source of p53 into cells, we triggered endogenous p53 expression by chemotherapeutics and UV (Fig. 3 and Fig. 4). We have employed more cell lines derived from various origins including CNE2 (nasopharyngeal), MCF-7 (breast), LoVo (colon), IMR-90 (normal lung fibroblast), PC-3 (prostate), and SK-OV-3 (ovary) to ascertain that the effect of p53 on MAD1 level is a general phenomenon. Down-regulation of MAD1 was again observed under these conditions, consistent with the results obtained by transfecting cells with p53-expression plasmid pCMVp53. We can only hypothesize that the discordance of our data from the SAGE results (39) might be due to a specific response of DLD-1 cells to p53 or technical issues involved in the SAGE analysis (73). In DLD-1 cells, the p53 gene has a 241F mutation, which has been shown to be defective for transactivation (74, 75). It is not fully understood whether and how the p53–241F mutant might interfere with the regulation of MAD1 by wild-type p53. During the preparation of this manuscript, an independent study on p53 regulation of MAD1 was published (76). In that study, wild-type p53 was found to be a poor activator of MAD1 promoter in HeLa, HCT116, and HCT116 p53–/− cells. However, a tumor-promoting gain-of-function p53 mutant (p53–281G) exhibited a distinctly potent transactivating activity on MAD1. Taken together with our findings that wild-type p53 represses MAD1, we postulate that the subversion of a p53 regulatory function on MAD1 promoter by some gain-of-function p53 mutants such as p53–281G might contribute to their tumor-promoting abili-
ties. In this regard, it will be of great interest to see whether the p53–241F mutant in DLD-1 cells might have such activity.

One might postulate two mechanisms by which p53 regulates MAD1, protein degradation or transcriptional repression. By taking advantage of proteasome inhibitors, we sought to rule in or rule out the first possibility. If p53 promotes ubiquitination of MAD1, the proteasome inhibitors would block the degradation activity, and the protein level of MAD1 would not decrease even in the presence of p53. Nevertheless, addition of proteasome inhibitors to cisplatin-treated cells did not elevate the MAD1 protein level from repressive to normal status. p53 maintained its repressive activity on MAD1 protein expression (Fig. 3). These results, in keeping with data from promoter maintained its repressive activity on MAD1 protein expression correlated with transcription levels, in that histone deacetylation may repress transcription by intensifying DNA-histone tail interactions and hence blocking the access of transcriptional modulators to the DNA template or by removing the acetyl groups on histone tails that are crucial to the association of transcriptional modulators with chromatin. A number of transcriptional co-regulators are histone acetylases or histone deacetylases (58). HDACs thus play important roles in gene regulation by being a family of enzymes catalyzing the removal of acetyl groups from post-translationally acetylated proteins. Furthermore, acetylation of p53 activates transcription through recruitment of co-activators/histone acetyltransferases to the promoters of p53-responsive genes (82). These mechanisms may also contribute to the de-repression of MAD1 promoter by p53. Nevertheless, it is of interest to determine the acetylation level of p53 in the context of MAD1 promoter thereby presents a novel p53-responsive element that is distinct from the known p53 consensus binding site (7) or other thus far known sequences required for p53 repression. Whether specific nucleotide within this 38-bp p53-responsive element is crucial for p53 repression remains unknown, and further deletion or mutation of specific nucleotides may help elucidate this issue.

p53 Represses MAD1 Expression through HDAC-mSin3a—We showed that the repression of MAD1 by p53 was alleviated when HDAC inhibitor TSA was added to the cells (Fig. 8). Histone acetylation levels are generally believed to be correlated with transcription levels, in that histone deacetylation may repress transcription by intensifying DNA-histone tail interactions and hence blocking the access of transcriptional modulators to the DNA template or by removing the acetyl groups on histone tails that are crucial to the association of transcriptional modulators with chromatin. A number of transcriptional co-regulators are histone acetylases or histone deacetylases (58). HDACs thus play important roles in gene regulation by being a family of enzymes catalyzing the removal of acetyl groups from post-translationally acetylated proteins. Furthermore, acetylation of p53 activates transcription through recruitment of co-activators/histone acetyltransferases to the promoters of p53-responsive genes (82). These mechanisms may also contribute to the de-repression of MAD1 promoter by p53. HDAC1 interacts with the co-repressor mSin3A to form a repression complex (83) which is recruited to specific promoters via association with transcription factors such as Sp1/3 (84) and E2F (85). Transcriptional repression of MAP4, stathmin, and BCL12 by p53 has been demonstrated to be mediated by HDAC1 and mSin3A (10, 61). Our results from the ChIP assay prominently illustrated that p53, HDAC1, and mSin3A proteins bind to MAD1 promoter in vivo in Hep3B cells transfected with p53 (Fig. 9). Remarkably, neither HDAC1 nor mSin3A associated with MAD1 promoter when p53-deficient Hep3B
p53 Represses MAD1 Expression

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