[CANCER RESEARCH 57, 5013–5016, November 15, 1997]

Advances in Brief

Mdm-2 Phosphorylation by DNA-dependent Protein Kinase Prevents Interaction with p53[1]

Lindsey D. Mayo, John J. Turchi, and Steven J. Berberich[2]

Department of Biochemistry and Molecular Biology, Wright State University, Dayton, Ohio 45435

Abstract

In response to genotoxic stress, the p53 tumor suppressor protein exerts a G_1 cell cycle arrest that is dependent on its ability to transactivate downstream target genes. This p53-dependent G_1 block is reversed by the binding of Mdm-2 to p53, preventing further transactivation. Interestingly, following DNA damage, the *mdm-2* gene is also transcriptionally activated by p53, and therefore, the question of how p53 can continue to transactivate genes in the presence of its own negative regulator has remained unanswered. Here, we provide evidence that phosphorylation of Mdm-2 protein by DNA-dependent protein kinase (DNA-PK) blocks its ability to associate with p53 and regulate p53 transactivation. The data support a model by which DNA-PK activation by DNA damage and phosphorylation of Mdm-2 renders the Mdm-2 protein unable to inhibit p53 transactivation, resulting in cell cycle arrest. Following DNA repair, the loss of DNA-PK activity results in newly synthesized Mdm-2 protein that is unphosphorylated and, therefore, capable of binding to p53, allowing cell cycle progression.

Introduction

In response to a number of DNA-damaging agents, p53 has been shown to concurrently transactivate genes responsible for cell cycle arrest (*p21*, *gadd45*, and *cyclin G*) and the *mdm-2* gene, which encodes a protein that is capable of binding to and inactivating p53 function (1, 2). We wished to explore the effects of posttranslational modifications on the p53-Mdm-2 interaction in an effort to discern how p53 transactivation might continue in the presence of elevated Mdm-2 protein. One posttranslational modification with the requisite activity to effect p53-Mdm-2 association is phosphorylation by DNA-PK.[3] DNA-PK is a nuclear serine/threonine protein kinase with the unique property that its activity is dependent on DNA discontinuities. DNA-PK has been implicated in both double-stranded DNA break repair and recombination (3). In fact, mutations in the Ku subunits, which are responsible for DNA binding, or the catalytic subunit, DNA-PKcs, result in radiosensitivity in humans and severe combined immune deficiency syndrome in mice (4–7). DNA-PK has been previously shown to phosphorylate a number of nuclear proteins *in vitro*, including p53 (8). Although DNA-PK phosphorylation of p53 may affect the stabilization of p53 (9), p53 DNA-PK mutants still possess the ability to transactivate *p53* response genes (10).

Materials and Methods

Protein Purification. A prokaryotic expression vector (pRSETA), containing the human *mdm-2* or p53 cDNAs, was electroporated into *Escherichia coli* strain BL21DE3 for protein expression. Induction of the histidine-tagged fusion proteins was performed as described by manufacturer (Qiagen). DNA-PK was purified to homogeneity from HeLa whole-cell extracts and used in *in vitro* reactions according to our previously published protocols (11).

ELISAs. DNA-PK phosphorylation reactions in the presence of ATP (PK) or an nonhydrolyzable analogue, AMPPNP, were performed on recombinant human p53, full-length Mdm-2, and S17A. Wells were coated with 50 ng of p53 and blocked with 1% BSA in a phosphate buffer. Mdm-2 (wild-type or S17A) protein (10 ng) was allowed to incubate with bound p53 for 1 h. A Mdm-2 monoclonal antibody (SMP14; Santa Cruz Biotechnology) followed by the addition of a goat antimouse horseradish peroxidase-conjugated antibody was used to detect Mdm-2 protein that was bound to p53. Using 3, 3′ 5, 5′ tetramethylbenzidine base as a substrate for color development, the V_{max} of the horseradish peroxidase enzyme was determined by quantitation of $A_{610\ nm}$ using Soft Max Pro software.

Site-directed Mutagenesis. Human *mdm-2* serine-17 was converted to alanine using two complementary oligonucleotides (5′-GCTGTAACCACCGCACAGATTCCAGC-3′ and 5′-GCTGGAATCTGTGCGGTGGTTACAGC-3′), with a bp change of T → G. The site-directed mutagenesis was completed using Pfu polymerase (Stratagene). After amplification, the reaction was digested with restriction enzyme *DpnI* to linearize parental strand DNA and transformed into maximum competent DH5α cells (Life Technologies, Inc.). Confirmation of mutagenesis was completed by dideoxy DNA sequencing.

CAT Assays. H1299 cells were plated at 1×10^5 and incubated overnight at 37°C in DMEM plus 10% fetal bovine serum. Cells were transfected with a total of 8 μg of supercoiled plasmid DNA using Lipofectamine (Life Technologies, Inc.). Forty-eight h, later the cells were harvested, and cell extracts were prepared using the freeze-thaw extraction method (12). To determine extract concentration, protein determinations were performed using the Bradford assay (Bio-Rad). CAT activity was measured by incubating each extract (~50 μg) with 50 μCi of [^{14}C]chloramphenicol and 5 mM *n*-butyl-CoA in 0.25 M Tris-Cl (pH 8.0) for 1–2 h. Acetylated products were purified by extraction with mixed xylenes and quantitated using liquid scintillation. Each CAT assay sample was performed in triplicate. The Western assay was performed as described previously (13), except for the following modifications. One hundred μg of H1299 extracts were resolved on a 8% SDS-polyacrylamide gel, transferred to a polyvinylidene fluoride membrane, and probed with a Mdm-2 monoclonal antibody (SMP-14; Santa Cruz Biotechnology).

Results

On the basis of an amino acid analysis of the full-length, human Mdm-2 protein, eight potential DNA-PK sites (9) were identified (Fig. 1*A*). To determine whether Mdm-2 could be phosphorylated by DNA-PK, *in vitro* phosphorylation reactions were performed with recombinant Mdm-2 protein, purified DNA-PK, and [γ-^{32}P]ATP. The data presented in Fig. 1*B* demonstrated that, in the absence of DNA (*Lane 1*), no DNA-PK phosphorylation of Mdm-2 was observed. However, in the presence of DNA (*Lanes 2* and *3*), Mdm-2 was effectively phosphorylated by DNA-PK.

One specific DNA-PK phosphorylation site on Mdm-2 was local-

Received 8/25/97; accepted 10/3/97.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by NIH Grants CA64374 (to J. J. T.) and CA64430 (to S. J. B.) and by the Ohio Cancer Research Association (to S. J. B.). L. D. M. was supported by a predoctoral fellowship from the Wright State University Biomedical Sciences Program.

[2] To whom requests for reprints should be addressed. Phone: (937) 775-4494; Fax: (937) 775-3730; E-mail: sberberich@wright.edu.

[3] The abbreviations used are: DNA-PK, DNA-dependent protein kinase; CAT, chloramphenicol acetyltransferase.

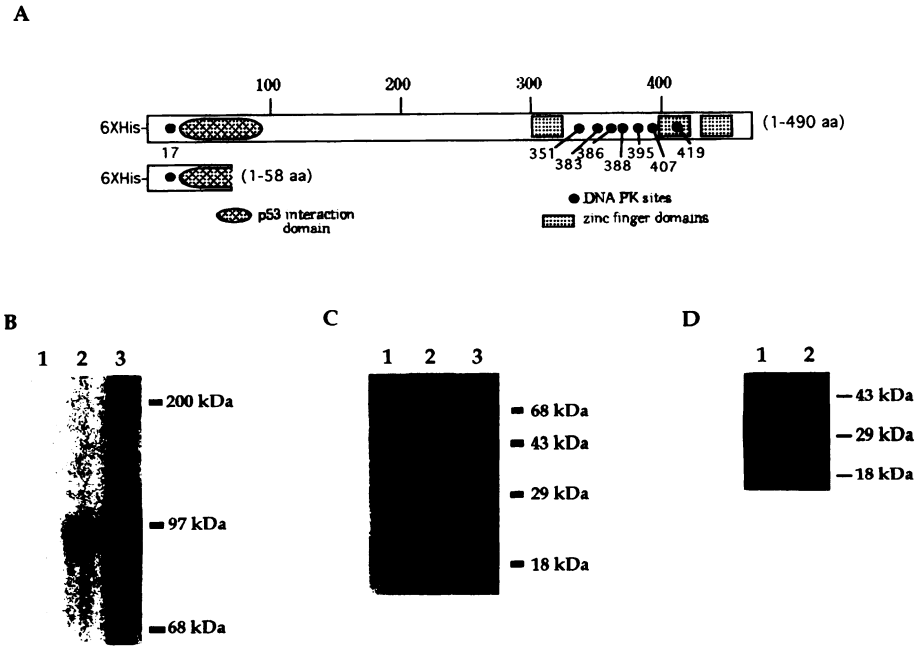

Fig. 1. DNA-PK phosphorylation of recombinant Mdm-2 protein. *A*, schematic of full-length Mdm-2 and a truncated peptide containing amino acids 1–58, defining the potential sites for DNA-PK phosphorylation (*numbers*) with respect to structural domains. *B*, DNA-PK reactions were performed as described previously (11). *Lane 1*, 100 ng of histidine-tagged recombinant Mdm-2 protein in the absence of activated calf thymus DNA; *Lanes 2* and *3*, activated DNA with 30 and 100 ng, respectively, of Mdm-2. *C*, NH_2-terminal Mdm-2 peptide was used in a DNA-PK phosphorylation reaction in the absence (*Lane 1*) or presence (*Lane 2*) of activated DNA. *Lane 3*, site-directed Mdm-2 mutant in which Ser-17 was changed to an alanine was subjected to a DNA-PK phosphorylation reaction. *D*, casein kinase II phosphorylation reactions were performed on truncated Mdm-2 (*Lane 1*) or S17A (*Lane 2*) to verify the presence of equivalent amounts of recombinant protein.

ized by site-directed mutagenesis. Interestingly, of the eight potential DNA-PK sites located on Mdm-2, only Ser-17 was adjacent to the p53 interaction domain (Fig. 1*A*). Therefore, a serine-to-alanine mutation at this position was created and expressed in bacteria as a truncated polypeptide consisting of amino acids 1–58 of Mdm-2. A control 58-amino acid polypeptide of Mdm-2, possessing the wild-type sequence, was also prepared, and both wild-type and S17A proteins were assayed for *in vitro* phosphorylation by DNA-PK (Fig. 1*C*). Although the wild-type 58-amino acid Mdm-2 polypeptide was phosphorylated by DNA-PK in a DNA-dependent manner (*Lanes 1* and *2*), the S17A polypeptide could not be phosphorylated by DNA-PK (*Lane 3*). We used a casein kinase II phosphorylation of Mdm-2 (wild-type and S17A) to ensure equal amounts of each truncated protein. The results in Fig. 3*D* demonstrated equal signal intensities of the two Mdm-2 proteins when they were phosphorylated by casein kinase II. The ability of a full-length S17A Mdm-2 protein to be phosphorylated by DNA-PK (data not shown) provides evidence that additional DNA-PK site(s) are located in the COOH region of Mdm-2. Taken together, these results clearly indicate that Ser-17 of the human Mdm-2 protein is a target for DNA-PK phosphorylation.

Having demonstrated that DNA-PK phosphorylated human Mdm-2 specifically at Ser-17, we designed a series of experiments to determine whether this phosphorylation site had an effect on the ability of Mdm-2 to interact with p53. A modified ELISA was used to assess p53-Mdm-2 interactions following DNA-PK phosphorylation of each protein. DNA-PK phosphorylation of Mdm-2 resulted in a dramatic decrease (>90%) in the amount of Mdm-2 associated with recombinant p53 (Fig. 2*A*). In sharp contrast, DNA-PK phosphorylation of p53 resulted in only a modest decrease in the amount of unphosphorylated Mdm-2 that was bound to p53. Consistent with Mdm-2 phosphorylation being the critical determinant to p53-Mdm-2 association, the phosphorylation of both proteins resulted in a decrease interaction comparable to that seen with Mdm-2 phosphorylation alone (Fig. 2*A*).

To ascertain the DNA-PK phosphorylation site on Mdm-2 that inhibits its ability to interact with p53, a full-length Mdm-2 protein possessing the S17A mutation was analyzed by ELISA. The unphosphorylated S17A retained its ability to bind to p53 at levels that were comparable to those of wild-type Mdm-2. However, DNA-PK phosphorylation of S17A failed to elicit the decrease in p53-Mdm-2 interaction that was seen with DNA-PK-phosphorylated Mdm-2 (Fig. 2*B*). Also, p53-S17A complex formation could not be decreased by DNA-PK phosphorylation of p53 alone or when both proteins were phosphorylated (Fig. 2*B*). Thus, the *in vitro* results point to Ser-17 as

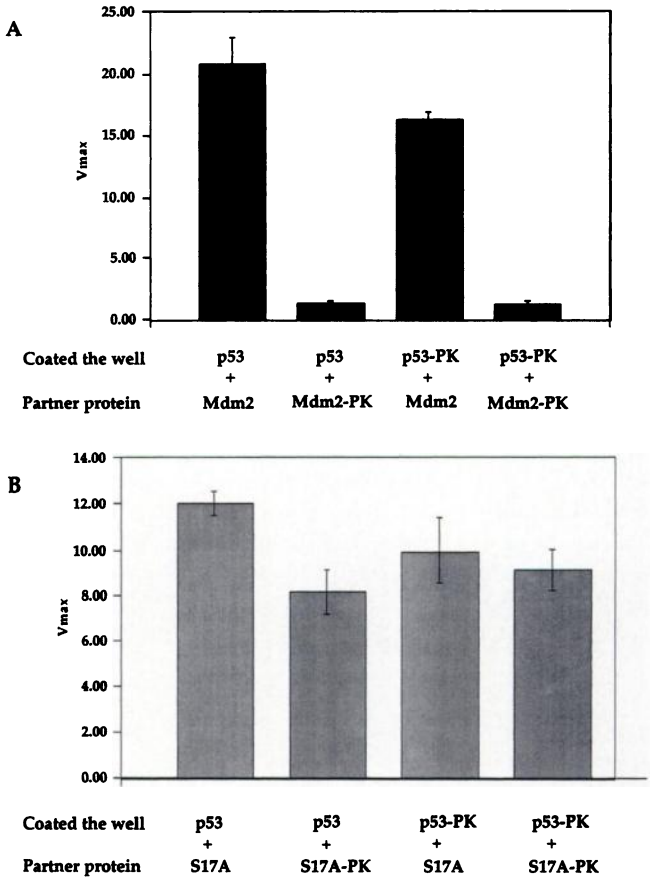

Fig. 2. The effects of Mdm-2 phosphorylation by DNA-PK on the interaction with p53 using a modified ELISA. *A*, comparison of the effects of DNA-PK phosphorylation of Mdm-2 on its interaction with p53. *B*, comparison of S17A with p53. *Columns*, V_{max} calculations for triplicate samples taken for each experimental condition; *bars*, SD.

a DNA-PK phosphorylation site on human Mdm-2 that is capable of regulating p53-Mdm-2 association.

Given that Mdm-2 phosphorylation by DNA-PK at Ser-17 alters its ability to bind p53, we set out to assess how this posttranslation modification of Mdm-2 would affect p53-dependent transactivation *in vivo*. H1299 cells that lack p53 protein (14) were transfected with a *p53CAT* reporter gene either alone, with a p53 expression vector, or with p53 and *mdm-2* expression vectors. The p53 transactivation potential was determined by monitoring CAT activity. Addition of the p53 expression vector produced a 12-fold increase in CAT activity (Fig. 3). Upon the addition of wild-type and S17A *mdm-2* expression vectors, p53 transactivation decreased 43% and 80%, respectively. This increased reduction in p53 transactivation by the S17A *mdm-2* expression vector is not the result of differences in the amount of Mdm-2 protein produced because Western analysis demonstrated equivalent Mdm-2 protein levels (Fig. 3, *inset*) and has been reproduced in cells lines possessing wild-type p53 protein (data not shown). One interpretation of the p53 transactivation data in Fig. 3 is that the inability to phosphorylate the S17A protein *in vivo* allows it to block p53 transactivation more effectively than did the DNA-PK-phosphorylatable wild-type Mdm-2 protein. Consistent with this interpretation, the process of mammalian cell transfection has been demonstrated to trigger a p53-dependent cell cycle arrest (15) and, thus, may also serve to activate DNA-PK.

Discussion

The data presented in this report are consistent with a model (Fig. 4) whereby DNA-PK phosphorylates the human Mdm-2 protein at Ser-17, effectively preventing Mdm-2 from complexing with p53. Given that DNA-PK activity is dependent on double-stranded DNA breaks, Mdm-2 phosphorylation at Ser-17 could explain how p53 transactivates the *mdm-2* gene but is not initially inhibited by the resulting elevated Mdm-2 protein. This posttranslational modification of Mdm-2 would allow further p53 transactivation of genes, which is required for cell cycle arrest (Fig. 4*A*). Then, upon the elimination of DNA damage, the concurrent decrease in DNA-PK activity would create an increase in unphosphorylated Mdm-2 protein and favor the formation of p53-Mdm-2 complexes (Fig. 4*B*). Combined with the recent observations that Mdm-2 association with p53 results in the rapid degradation of p53 (16, 17), our results suggest a mechanism by which the cell can effectively regulate the timing of p53 degradation.

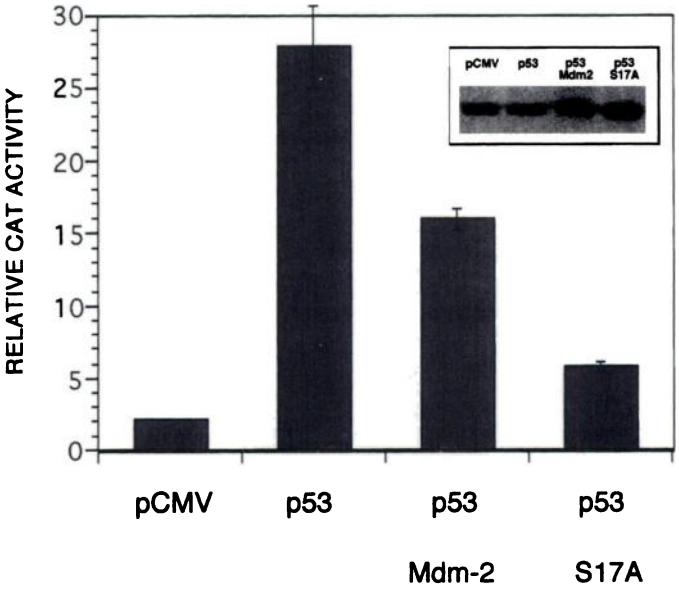

Fig. 3. p53 transactivation is inhibited by S17A Mdm-2. H1299 cells were transfected as described in "Materials and Methods." All transfections contained 1 μg of $PG_{13}CAT$, a p53 reporter gene (18). The pCMV transfection also contained 7 μg of pCMV. Each p53 transfection contained 2 μg of p11-4, a murine p53 expression vector (19). The Mdm-2 and S17A transfections contained 5 μg of pCMV*mdm-2* or pCMVS17A, respectively. *Columns*, relative CAT in milliunits of CAT/μg of protein for triplicate samples taken for each experimental condition; *bars*, SD. *Inset*, Western blot analysis to determine Mdm-2 protein levels in transfected H1299 cells. *Band*, full-length Mdm-2 protein (p90).

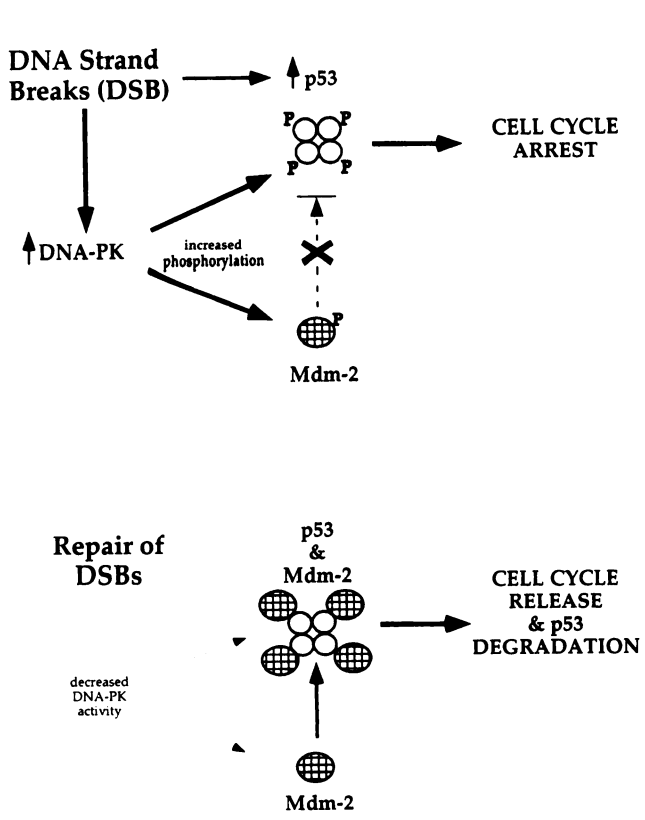

Fig. 4. Model predicting how DNA-PK phosphorylation of Mdm-2 inhibits p53-Mdm-2 interaction following DNA damage. *A*, in response to double-stranded DNA breaks, DNA-PK activity and p53 protein levels increase. This, in turn, results in phosphorylation of both Mdm-2 and p53 proteins. The phosphorylation of Mdm-2 at Ser-17 effectively blocks its ability to interact with p53. p53 is able to continue to transactive genes responsible for cell cycle arrest. *B*, following the repair of damaged DNA, DNA-PK activity is eliminated and the newly synthesized Mdm-2 protein, now unphosphorylated, binds p53 and target p53 for degradation (16, 17).

Acknowledgments

We would like to thank Dr. Bert Vogelstein for the human *mdm2* cDNA and $PG_{13}CAT$ plasmid vectors and Dr. Dale Haines for the H1299 cells.

References

1. Perry, M. E., Piette, J., Zawadzki, J. A., Harvey, D., and Levine, A. J. The *mdm-2* gene is induced in response to UV light in a p53-dependent. Proc. Natl. Acad. Sci. USA, *90:* 11623–11627, 1993.
2. Chen, C-Y., Oliner, J. D., Zhan, Q., Fornance, A. J., Vogelstein, B., and Kastan, M. B. Interactions between p53 and MDM2 in a mammalian cell cycle checkpoint pathway. Proc. Natl. Acad. Sci. USA, *91:* 2648–2688, 1994.
3. Jackson, S. P., and Jeggo, P. A. DNA double-strand break repair and V(D)J recombination: involvement of DNA-PK. Trends Biochem. Sci., *20:* 412–415, 1995.
4. Boubnov, N. B., Hall, K. T., Wills, Z., Lee, S. E., He, D. M., Benjamin, D. M., Pulaski, C. R., Band, H., Reeves, W., Hendrickson, E. A., and Weaver, D. T. Complementation of the ionizing radiation sensitivity, DNA end binding and V(D)J recombination defects of double-strand break repair mutants by the p86 Ku autoantigen. Proc. Natl. Acad. Sci. USA, *92:* 890–894, 1995.
5. Danska, J. S., Holland, D. P., Mariathasan, S., Williams, K. M., and Guidos, C. J. Biochemical and genetic defects in the DNA-dependent protein kinase in murine scid lymphocytes. Mol. Cell. Biol., *16:* 5507–5517, 1996.
6. Finnie, N. J., Gottlieb, T. M., Blunt, T., Jeggo, P. A., and Jackson, S. P. DNA-dependent protein kinase defects are linked to deficiencies in DNA repair and V(D)J recombination. Philos. Trans. R. Soc. London Ser. B Biol. Sci., *351:* 173–179, 1996.

7. Lees-Miller, S. P., Godbout, R., Chan, D. W., Weinfeld, M., Day, R. S. I., Barron, G. M., and Allalunis-Turner, J. Absence of p350 subunit of DNA-activated protein kinase from a radiosensitive human cell line. Science (Washington DC), *267:* 1183–1185, 1995.
8. Anderson, C. DNA damage and the DNA-activated protein kinase. Trends Biochem. Sci., *18:* 433–437, 1993.
9. Lees-Miller, S. P., Sakaguchi, K., Ullrich, S. J., Appella, E., and Anderson, C. W. Human DNA-activated protein kinase phosphorylates serines 15 and 37 in the amino-terminal transactivation domain of human p53. Mol. Cell. Biol., *12:* 5041–5049, 1992.
10. Fuchs, B., O'Connor, D., Fallis, L., Scheidtmann, K. H., and Lu, X. p53 phosphorylation mutants retain transcription activity. Oncogene, *10:* 789–793, 1995.
11. Turchi, J. J., and Henkels, K. Human Ku autoantigen binds cisplatin-damaged DNA but fails to stimulate human DNA-activated protein kinase. J. Biol. Chem., *271:* 13861–13867, 1996.
12. Rosenthal, N. Identification of regulatory elements of cloned genes with functional assays. Methods Enzymol., *152:* 704–722, 1987.
13. Mayo, L. D., and Berberich, S. J. Wild type p53 protein is unable to activate the *mdm-2* gene during F9 cell differentiation. Oncogene, *13:* 2315–2321, 1996.
14. Funk, W. D., Pak, D. T., Karas, R. H., Wright, W. E., and Shay, J. W. A transcriptionally active DNA-binding site for human p53 protein complexes. Mol. Cell. Biol., *12:* 2866–2871, 1992.
15. Renzing, J., and Lane, D. P. p53-dependent growth arrest following calcium phosphate-mediated transfection of murine fibroblasts. Oncogene, *10:* 1865–1868, 1995.
16. Haupt, Y., Maya, R., and Oren, M. Mdm2 promotes the rapid degradation of p53. Nature (Lond.), *387:* 296–299, 1997.
17. Kubbutat, M. H. G., Jones, S. N., and Vousden, K. H. Regulation of p53 stability by Mdm2. Nature (Lond.), *387:* 299–303, 1997.
18. Kern, S. E., Pientenpol, J. A., Thiagalingam, S., Seymour, A., Kinzler, K. W., and Vogelstein, B. Oncogenic forms of p53 inhibit p53-regulated gene expression. Science (Washington DC), *256:* 827–830, 1992.
19. Tan, T., Wallis, J., and Levine, A. J. Identification of the p53 protein domain involved in formation of the simian virus 40 large T-antigen p53 protein complex. J. Virol., *59:* 574–583, 1986.