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*Advances in Brief*

# Increases in Sequence Specific DNA Binding by *p53* following Treatment with Chemotherapeutic and DNA Damaging Agents[1]

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## Abstract

**We have investigated the effect of chemotherapeutic and DNA damaging agents on binding of the tumor suppressor phosphoprotein *p53* to its consensus DNA sequence. Activation of *p53*-DNA binding was seen for treatment with radiation, hydrogen peroxide, actinomycin D, Adriamycin, etoposide, camptothecin, 5-fluorouracil, mitomycin C, and cisplatin. These results showed that DNA strand breaks were sufficient to lead to increased levels of *p53*. The protein synthesis inhibitor cycloheximide blocks the increase in *p53* following DNA damage. The increase in *p53* activation in camptothecin treated cells may result, at least in part, from an increased half-life of the protein and consequent increases in intracellular protein concentration.**

## Introduction

The nuclear phosphoprotein *p53* is a tumor suppressor gene involved in cell cycle control (1–4). Mutations of the *p53* locus are among the most common changes seen in human malignancies (5). Loss of wt*p53*,[3] either by deletion or point mutation, increases the susceptibility to tumor formation. This has been observed in patients with inherited defects in the *p53* alleles (Li-Fraumeni syndrome; Ref. 6) and experimentally in transgenic mice with homozygous deletions of the *p53* gene (7). In addition to its established role in the development of malignancy, recent studies indicate that levels of *p53* increase rapidly in response to DNA damage induced by ionizing radiation (1). Cells which incur DNA damage exhibit delays at both the $G_1$ and $G_2$ phases of the cell cycle, and these cell cycle delays are thought to allow cells to repair DNA before continuing to DNA synthesis or mitosis. Increased wt*p53* levels are required to initiate the $G_1$ block, suggesting that wt*p53* is a component of the signal transduction pathway leading to a $G_1$ arrest (3). In contrast, cells expressing mutant *p53* do not block in $G_1$ following DNA damage (1). Since mutations in the *p53* gene abolish its ability to bind DNA, it is thought that wt*p53* initiates $G_1$ arrest through its sequence specific DNA binding.

Among the questions raised by this work, with potential implications for both DNA damage detection mechanisms and therapeutics, are which agents and what types of DNA damage lead to increases in the levels of wt*p53*? The presumed reason for the cell cycle block following DNA damage is to allow time for repair. Consequently, activation of mechanisms for DNA repair may be a component of the DNA damage response and thus will influence how the cell responds to and repairs DNA damage from chemotherapeutic agents. A system involving *p53* may be important or at least serve as a model for cellular responses to DNA damage which might be therapeutically significant. A high percentage of human cancers have a mutant *p53* and its presence or absence may influence the efficacy of specific therapies. In addition, determining which particular agents increase wt*p53* may yield information relating to the DNA lesion which initiates the wt*p53* signal transduction pathway.

There is substantial evidence that wt*p53* acts as a regulator of transcription and exerts its physiological effect via sequence specific DNA binding (8–11). Consequently, studying the DNA-binding functions of wt*p53* may provide important information on the physiological function of wt*p53*. A number of specific DNA sequences to which wt*p53* binds have been identified in genomic DNA (8, 10) and from *in vitro* studies of binding of wt*p53* to random oligonucleotides (9). Using these oligonucleotides in conjunction with nuclear extracts and specific *p53* antibodies, we have developed a sensitive EMSA assay to detect the presence of endogenous wt*p53*. Using this assay, we assessed how DNA damaging agents altered the levels of wt*p53*-DNA binding.

## Materials and Methods

**Cells.** NIH-3T3 mouse fibroblasts were grown in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 10% bovine calf serum (HyClone, Los Angeles, CA), L-glutamine, and penicillin/streptomycin (Gibco, Grand Island, NY). Cells were cultured on 100-mm dishes and used when 80% or more confluent.

**Preparation of Nuclear Extract.** Following drug exposure or irradiation of the cells, the medium was aspirated and the cells were washed twice with phosphate buffered saline at 4°C. Nuclear extracts were prepared as described (9) with the following modifications: cells were lysed by addition of 2.5 ml of Buffer A (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.6-20% glycerol-10 mM NaCl-1.5 mM $MgCl_2$-0.2 mM EDTA-1 mM dithiothreitol-1 mM phenylmethylsulfonylfluoride-0.1% Triton X-100), scraped from the dish using a rubber scraper, resuspended, and incubated on ice for 5 min. Lysates were centrifuged (800 *g* for 4 min) and the nuclear pellet was resuspended in 3 volumes of extraction buffer (Buffer A plus 500 mM NaCl). Nuclei were incubated at 4°C for 30 min with continuous agitation and then centrifuged at 35,000 *g* for 10 min. The supernatant containing *p53* was removed for immediate use or aliquoted and stored at −80°C for subsequent assays.

**Electrophoretic Mobility Shift Assays.** The consensus *p53* binding sequence determined by Funk (GGACATGCCCGGGCATGTCC; Ref. 9) was synthesized, prepared in double stranded form, and labeled as previously described (12). Binding reactions consisted of nuclear extract (20 μg of protein), $^{32}$P-labeled oligonucleotide (0.5 ng), salmon sperm DNA (1 μg; Sigma), and *p53* antibody (100 ng pAb421; Oncogene Science, Manhasset, NY) with Buffer A (without the Triton) used to reach a final volume of 25 μl. Binding reactions were incubated at room temperature for 20 min and 8 μl were analyzed on 4% nondenaturing polyacrylamide gels as previously described (12).

**DNA Damaging Agents.** All agents were obtained from Sigma and stored at −20°C after preparation, unless otherwise noted. High concentrations of camptothecin precipitated when added to medium. To avoid this, medium was rapidly added to the appropriate quantity of camptothecin solution and then added to the culture dish. All other compounds were added directly to culture dishes. Stock solutions were prepared as follows: 0.25 mg/ml actinomycin D in 95% ethanol; 2.5 mg/ml Adriamycin in phosphate buffered saline; 1 mg/ml cisplatin in $H_2O$, 10 mM camptothecin in DMSO, 10 mM etoposide in DMSO,

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[3] The abbreviations used are: wt*p53*, wild-type *p53*; EMSA, electrophoretic mobility shift assay(s); DMSO, dimethyl sulfoxide; *gadd*, growth arrest and DNA damage.

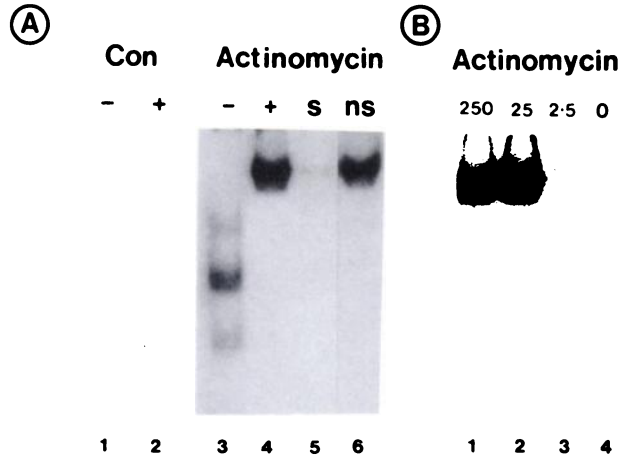


Fig. 1. Effect of actinomycin D on the binding of *p53* to its consensus sequence in the presence and absence of Ab421. In *A*, nuclear extracts from untreated control (*Con*, *Lanes 1* and *2*) or actinomycin treated cells (250 ng/ml for 3 h; *Act*, *Lanes 3–6*) were prepared as described in "Materials and Methods." Extracts were mixed with $^{32}$P-*p53* oligonucleotide in the absence (*Lanes 1* and *3*) or presence (*Lanes 2*, *4-6*) of Ab421 (100 ng). For competition assays, binding reactions were supplemented with 100-fold excess of unlabeled *p53* oligonucleotide (*Lane 5*) or a 100-fold excess of a nonspecific oligonucleotide (*Lane 6*). In *B*, cells were exposed to 250 ng/ml (*Lane 1*), 25 ng/ml (*Lane 2*), or 2.5 ng/ml (*Lane 3*) actinomycin D for 3 h or were untreated (*Lane 4*). Nuclear extracts were prepared and EMSA was carried out to detect *p53*-DNA binding as described in "Materials and Methods." *s*, specific; *ns*, nonspecific.

50 mM 5-fluorouracil in $H_2O$, 1 mM iodoacetamide in $H_2O$, 1 mg/ml mitomycin C in $H_2O$, 100 μM taxol in DMSO. Hydrogen peroxide was prepared at a concentration of 100 mM in $H_2O$ on the day of the experiment. Cells were washed in Dulbecco's modified Eagle's medium without serum and incubated in the same prior to addition of the hydrogen peroxide. Radiation was delivered with a $^{137}$Cs irradiator at a dose rate of 100 cGy/min. Cycloheximide was prepared as a stock solution of 26 mM in DMSO and stored at −20°C.

## Results and Discussion

Fig. 1*A* illustrates EMSA binding of endogenous wt*p53* to its palindromic consensus DNA sequence for both untreated and actinomycin D treated cells. In the absence of antibody 421 (Fig. 1*A*, *Lane 1*) a number of *p53* oligonucleotide-protein complexes were detected in control cells. The addition of anti-*p53* antibody 421 (Ab421) revealed a supershifted band (Fig. 1*A*, *Lane 2*), indicating the presence of wt*p53* in the protein-DNA complexes. In cells treated with actinomycin D, a compound known to increase wt*p53* protein levels (1), Ab421 also revealed a supershifted Ab421-protein complex when compared with extracts without antibody (Fig. 1*A*, *Lanes 3* and *4*). The supershifted band in *Lanes 2* and *4* was seen only with Ab421 and could not be detected with antibodies raised against other portions of the *p53* protein.[4] Additionally, this supershifted band was greatly increased in actinomycin treated cells (compare Fig. 1*A*, *Lanes 2* and *4*). To further characterize the specificity of the interaction of the *p53* consensus sequence with wt*p53*, competition assays were carried out. A 100-fold molar excess of unlabeled *p53* oligonucleotide competitively inhibits *p53* binding (Fig. 1*A*, *Lane 5*, *s*) while an unrelated ("nonself") oligonucleotide had little effect on the *p53*-DNA complex (Fig. 1*A*, *Lane 6*, *ns*). The presence of, and changes associated with, the lower molecular weight nonspecific bands were variable between experiments. Fig. 1*A* demonstrates that specific *p53*-DNA complexes can be detected in nuclear extracts using an antibody specific for wt*p53* and that the levels of this DNA-protein complex can be altered by actinomycin D. This increase was examined further in Fig. 1*B* which indicates that *p53*-DNA binding increases in a dose-dependent manner for actinomycin D concentrations of 2.5 to 250 ng/ml using relatively short (3-h) exposure times.

[4] B. Price, unpublished observations.

In Fig. 2 the effect on *p53*-DNA binding activity of a range of chemotherapeutic and DNA damaging agents was studied. Increases in wt*p53* DNA binding activity was seen for the free radical inducing treatments (radiation, hydrogen peroxide), the combination DNA intercalaters/topoisomerase inhibitors (actinomycin, Adriamycin) and the topoisomerase inhibitors camptothecin and etoposide. The antimetabolite 5-fluorouracil also led to significant but less intense changes as did the alkylating antibiotic agent mitomycin C. Cisplatin treatment gave a small increase, whereas the alkylating agent iodoacetamide did not cause significant increases compared with controls. Concentrations of iodoacetamide one order of magnitude higher did not cause increases in *p53* levels/binding (data not shown). The concentrations used for studies in Fig. 2 were chosen to illustrate the efficacy of each agent, but increased *p53* levels also were observed in response to lower concentrations for most of the compounds, as illustrated for actinomycin D in Fig. 1*B*. For example, significant increases were seen for adriamycin at 0.1 μg/ml and for 5-fluorouracil at 10 μM. Included as a control is the microtubule active agent taxol which does not directly damage DNA but which will cause cell cycle arrest (13). Taxol caused a decrease in *p53*-DNA binding activity compared with untreated cells (Fig. 2). The response to taxol was variable, with other experiments showing slight increases or decreases relative to control (data not shown).

We examined the time course of activation of *p53* for cells treated with two agents operating by different mechanisms: the topoisomerase I inhibitor camptothecin; and the free radical producing agent $H_2O_2$. The time course of increase in *p53* following treatment with 100 μM camptothecin is shown in Fig. 3*A*. Increases in *p53*-DNA binding were detected as early as 30 min after camptothecin addition and continued for up to 6 h. Exposure of the cells to hydrogen peroxide leads to an increase over 2 h and then a decline to control levels after 6 h. It is unclear why $H_2O_2$ induction of *p53*-DNA binding should return to control levels after 2–4 h. Cellular metabolism of $H_2O_2$ may account for some of this decrease. However, transient activation of other transcription factors following peroxide treatment has been observed previously in NIH-3T3 cells (14). Previous studies have shown that increases in wt*p53* protein occur 30 min after irradiation, while for cells exposed to actinomycin there is a slow increase in wt*p53* protein over 24 h (1).

In Fig. 3*B* we have examined the inactivation of wt*p53*-DNA binding activity during recovery from three different stimuli. To do this, cells were exposed to either actinomycin D, camptothecin, or $H_2O_2$, followed by extensive washing to remove them (Fig. 3*B*). In actinomycin D treated cells, *p53*-DNA binding activity was slightly increased compared to the level at the beginning of the washout (Fig.

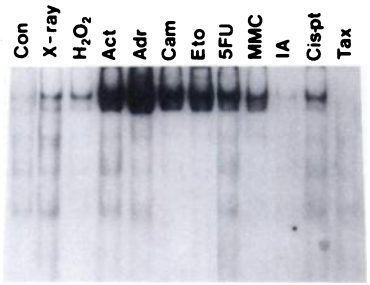


Fig. 2. Stimulation of *p53*-DNA binding by a range of chemotherapeutic/DNA damaging agents. Cells were treated as follows: *Con*, unstimulated controls; *X-ray*, radiation 5 Gy followed by 1 h recovery; $H_2O_2$, 500 μM for 2 h; actinomycin D (*Act*), 250 ng/ml, for 3 h; adriamycin (*Adr*), 10 μg/ml for 3 h; camptothecin (*Cam*), 100 μM for 3 h; etoposide (*Eto*), 400 μM for 3 h; 5-fluorouracil (*5FU*), 50 μM for 3 h; mitomycin C (*MMC*), 10 μg/ml for 24 h; iodoacetamide (*IA*), 10 μM for 3 h; cisplatin (*Cis-pt*), 20 μg/ml for 3 h; taxol (*Tx*), 10 nM for 4 h. Incubations were terminated at the indicated times, nuclear extracts were prepared, and EMSA were carried out as in "Materials and Methods."

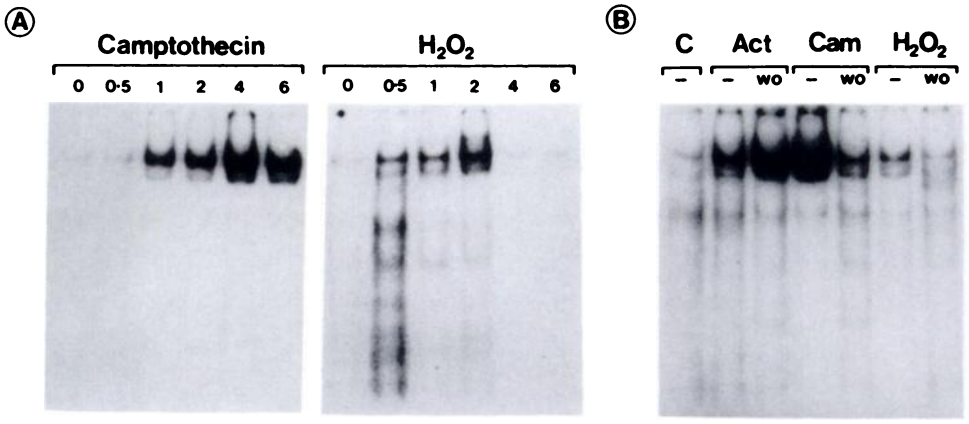


Fig. 3. Time course of activation and reversibility of *p53*-DNA binding activity. *A*, increase in *p53*-DNA binding as a function of time for camptothecin and $H_2O_2$. Cells were exposed to 100 μM camptothecin or 500 μM $H_2O_2$ for 0.5, 1, 2, 4, or 6 h or left untreated (*0*) before preparation of nuclear extracts and EMSA. In *B*, cells were incubated for 2 h in actinomycin (*Act*; 250 ng/ml), camptothecin (*Cam*; 100 μM), or $H_2O_2$ (500 μM). The washout procedure was to rinse cells in serum-free medium followed by fresh serum/Dulbecco's modified Eagle's medium and incubated for 1 h. Washes were repeated 4 times; –, sample prepared at completion of drug exposure; *wo*, washed.

3*B*). In contrast, when cells treated with camptothecin were washed free of the drug, a significant reduction in the levels of the *p53*-DNA binding activity (Fig. 3*B*) compared to that of unwashed cells (Fig. 3*B*) was seen. In $H_2O_2$ treated cells, *p53*-DNA binding activity returned to control levels after washout, as expected from previous data. These results are consistent with the known mechanisms of action of the three compounds. Actinomycin is a DNA intercalater whose binding is essentially irreversible, resulting in the chronic *p53* activation observed in these experiments. Camptothecin is a reversible inhibitor of topoisomerase I, the removal of which allows the topoisomerase to complete its catalytic cycle, leading to the observed decreases in *p53* activation. Hydrogen peroxide produces free radicals and single strand breaks in DNA. Once it is removed, DNA breaks may be repaired and the *p53* activity decreased to control levels. In addition, Fig. 3*A* showed that levels decreased at 6 h even if the hydrogen peroxide were left in place.

The above data demonstrate that the increase in *p53* binding activity can be reversed over the course of 4 h if the stimulus is removed. To determine if increased levels of w*tp53*-DNA binding seen here required new protein synthesis or activation of endogenous *p53*, cells were treated with the protein synthesis inhibitor cycloheximide. Fig. 4*A* indicates that exposure of unstimulated cells to cycloheximide eliminates any detectable *p53* signal (*Con* – and +). It also shows that pretreatment of the cells with cycloheximide inhibits completely the increases in *p53* caused by hydrogen peroxide, actinomycin, and camptothecin (Fig. 4*A*; compare *Lanes* – and + for each agent), three agents with different mechanisms for damaging DNA. This result indicates that new protein synthesis, presumably *p53* synthesis, is required in order for cells to increase levels of *p53*. The half-life of *p53*-DNA complex can be determined by adding cycloheximide to prevent new protein synthesis and measuring the levels of *p53* at various times after addition. Treatment of the unstimulated cells gives information on the decay of endogenous *p53*. In Fig. 4*A* (*Con* – and +) *p53*-DNA binding activity was undetectable 2 h after addition of cycloheximide, with an estimate of the half-life of binding of less than 30 min (data not shown). This is consistent with estimates of the half-life of w*tp53* protein previously obtained in unstimulated, untransformed NIH-3T3 cells and found to be about 20 min (15). Thus, the half-life of the binding of the *p53* to DNA in the presence of cycloheximide mirrors the half-life of the protein. The decay of the *p53*-DNA binding activity for camptothecin treated cells in the presence of cycloheximide is shown in Fig. 4*B* and indicates a half-life of hours (compare *Lane* 0 with *Lanes 2*, *4*, and *8*). There appears to be a relatively rapid decrease over the first 2 to 4 h but not much change between 4 and 8 h after cycloheximide addition. Thus, the nature of the decrease does not allow a precise half-life to be determined. Controls (Fig. 4*B*, *Lane 5*) treated with only camptothecin show higher levels of *p53*-DNA binding at 8 h than do cycloheximide treated cells. For comparison, untreated control cells have a much less intense signal (*e.g.*, compare Fig. 4*A*, *Lanes Con* – and *Cam* –). This

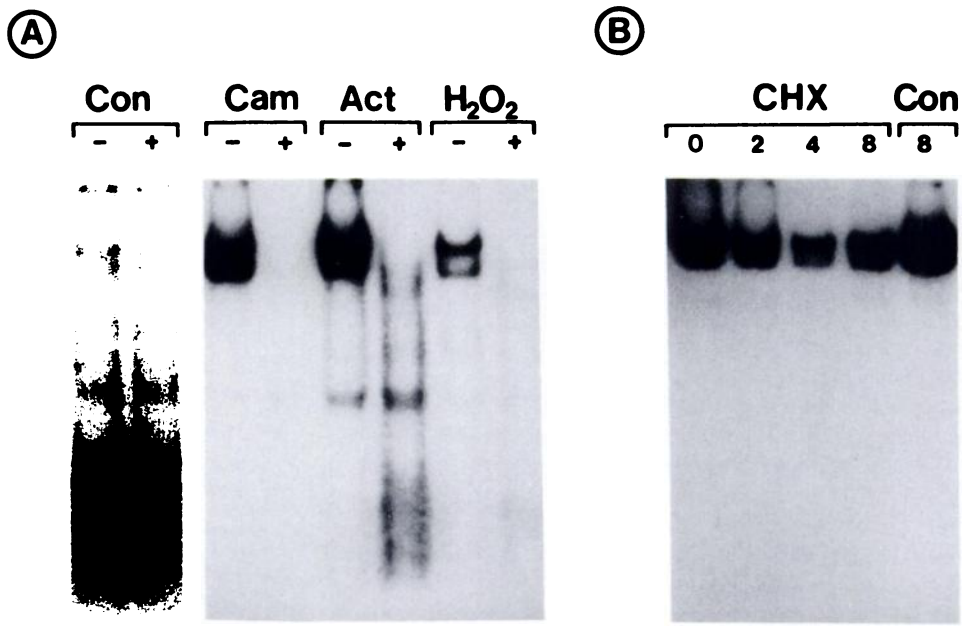


Fig. 4. Effect of cycloheximide on stimulation of *p53*-DNA binding. In *A*, cells were either untreated (–) or pretreated with 13 μM cycloheximide for 15 min (+) prior to addition of the specified compounds. Camptothecin (*Cam*; 100 μM) was added for 3 h, actinomycin D was added for 3 h (*Act*; 250 ng/ml), and $H_2O_2$ (500 μM) was added for 2 h. Cell extracts were then prepared for EMSA. Control cells (Con) were treated with cycloheximide for 2 h. In *B*, cells were pretreated with 100 μM camptothecin for 2 h prior to the addition of cycloheximide (13 μM). Nuclear extracts were prepared 0, 2, 4, or 8 h later for EMSA. Control (*Con*) cells were exposed to camptothecin but were not treated with cycloheximide.

clearly demonstrates an increase in the half-life of the *p53*-DNA complex (and therefore in the *p53* protein) in camptothecin treated cells.

The data in Fig. 1*A* demonstrate that DNA damaging agents such as actinomycin D induce *p53*-DNA binding, a response which may be secondary to increases in *p53* protein levels shown previously in ML-1 myeloblastic leukemia cells following actinomycin D or radiation treatment (1). The data indicate that EMSA can detect endogenous wt*p53* in nuclear extracts when used in combination with Ab421. Previous studies have noted that Ab421 may influence the interaction of wt*p53* with its DNA recognition site (9, 16). Ab421 interacts with the COOH-terminal region of *p53* (17), which is important in control of the activation of DNA binding. Removal of the 30 COOH-terminal amino acids leads to increased DNA binding and modifications of this portion of the protein may potentially play a role in *in vivo* regulation of *p53* (16). Use of *p53* antibodies recognizing other portions of the protein does not have the same effect on DNA binding and does not function in conjunction with EMSA (3, 9, 16).

Recent studies have examined the relationship between DNA damage and the multiple changes they induce in the cell including *gadd* gene expression, cell cycle arrest, and increased levels of wt*p53* (1–3). One aspect of these interrelated pathways that has not been addressed is what type of DNA damage causes induction of *p53*. Ionizing radiation, actinomycin D (1), UV radiation, and UV-mimetic treatments with 4-nitroquinolone-1-oxide (18) have all been shown to lead to increased levels of wt*p53*. The agents which we showed in Fig. 2 to increase *p53* levels cause DNA damage by a variety of mechanisms. γ-Radiation and peroxide cause DNA strand breaks via free radical formation or direct damage to the DNA (19). Actinomycin D and Adriamycin are DNA intercalaters which inhibit topoisomerase II (20, 21) and additional evidence indicates that actinomycin inhibits topoisomerase I as well (22). Etoposide is an inhibitor of topoisomerase II whereas camptothecin inhibits topoisomerase I (21). All of these agents will lead to strand breaks in DNA due to inhibition of topoisomerase activity. This indicates that single stranded DNA breaks are sufficient to cause increases in wt*p53* levels. Each agent leads to strand break formation by different mechanisms from that for radiation and $H_2O_2$ but lead to a similar effect on *p53* levels. 5-Fluorouracil acts via multiple pathways including incorporation into RNA, incorporation into DNA, and inhibition of DNA synthesis (20). The absence of a response to the alkylating agent iodoacetamide indicated that this particular type of damage does not induce the signal transduction pathway. This result is in agreement with previous data which showed that alkylating agents do not increase *p53* levels as had UV treatment in a BALB/c 3T3 cell line (18). However, both mitomycin C, which acts as an alkylating agent, and cisplatin, which acts as a DNA cross-linking agent (20), also cause increased levels of *p53*. These data demonstrate that, although single strand breaks are sufficient to cause increased *p53* levels, this is not the only type of DNA damage which leads to increases.

The spectrum of response to DNA damaging agents in terms of *p53* activation differs from other cellular response systems. This is not entirely unexpected since cells react to DNA damage via multiple pathways with distinct initiating events. This is important to note in light of the relationship that has been shown between *p53* activation and expression of the growth arrest gene, *gadd45*. A close correlation has been shown between activation of *p53* and *gadd45* expression (3). By contrast, in a study using HeLa cells with a CAT construct attached to the promoter of a different member of the *gadd* family of proteins, quite different agents induced *gadd153* expression (23). For example, the response to iodoacetamide was comparable to that seen for cisplatin, mitomycin C, Adriamycin, camptothecin, and etoposide (20–30 [times] control) and was roughly twice that seen for peroxide. In contrast, the response to radiation did not differ significantly from control. The inactivation of wild-type *p53* in HeLa cells may contribute to this marked difference, or it may be an indication that *gadd153* is stimulated by a different pathway to that involving *p53*/*gadd45*.

We have also studied the mechanism by which wt*p53* levels are increased. Inhibition of protein synthesis completely blocks the activation of *p53* and this suggests that new wt*p53* is synthesized in response to DNA damage. The decay of *p53* activity in camptothecin treated cells was greatly increased compared with that of controls, indicating that stabilization is at least one mechanism for the increased levels following DNA damaging agents. *p53* protein levels are increased in transformed cell lines mainly due to increases in the half-life of the protein (24). In SV40 transformed cells this was due in part to the association of the T-antigen with the *p53* protein (15). A previous study showed that for DNA damage induced with UV irradiation, *p53* levels were increased as well as the half-life (from 35 to 150 min; Ref. 18). These results are consistent with the data presented above for cells treated with chemotherapeutic agents.

In conclusion, we have demonstrated increased synthesis and binding of wt*p53* to its consensus DNA sequence after exposure to a range of DNA damaging agents. These data indicate that DNA strand breaks may be sufficient to cause increases in *p53* binding activity. Increases in the activation of wt*p53* appeared to be at least in part related to increases in the cellular concentration of protein. The alkylating agent iodoacetamide did not lead to increases, but cisplatin and mitomycin C did, suggesting that other, but not all, types of DNA damage may initiate this pathway. The class of DNA damaging agents which exert their activity by inducing single strand DNA breaks may activate a homeostatic mechanism in the cells which results in increased levels of wt*p53* bound to its specific DNA sites in the genome. This binding may serve to activate transcription of growth arrest genes (*e.g.*, *gadd45*; Ref. 3) and/or repress cell cycle genes and halt DNA replication.

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