Modulation of DNA Topoisomerase I Activity by *p53*

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ABSTRACT: The tumor suppressor protein *p53* plays a central role in the cellular response to genotoxic lesions and has been shown to be activated by most anticancer agents such as mitomycin C. We here show that mitomycin C treatment of human MCF7 breast adenocarcinoma cells results in increased topoisomerase I activity as measured by relaxation of supercoiled DNA and by phosphorylation of SR protein splicing factor. The increase in catalytic activity occurs in parallel with the nuclear accumulation of *p53,* resulting in detectable activation of topoisomerase I within less than 1 h of drug treatment. Furthermore, topoisomerase I co-immunoprecipitates with nuclear *p53,* suggesting that the activation of topoisomerase I may be a result of a direct interaction between the two proteins. *In vitro* experiments with purified recombinant proteins show that *p53* increases the catalytic activities of topoisomerase I as measured by relaxation of supercoiled DNA, stabilization of the covalent topoisomerase I−DNA complex (in the presence of camptothecin), and phosphorylation of SR protein splicing factor ASF/SF2. Furthermore, topoisomerase I sediments at a higher molecular weight in the presence of *p53* as revealed by sucrose density gradient analysis in the absence of DNA. Finally, *p53* modifies the thermal stability of topoisomerase I, protecting it from heat denaturation. Taken together, our results show that topoisomerase I and *p53* form molecular complexes *in vitro* as *in vivo,* and we suggest that the *p53*-mediated response to DNA damage may, at least in part, involve activation of topoisomerase I.

Eukaryotic DNA topoisomerase I alters DNA topology by transiently breaking one strand of DNA, passing the other strand through the break, and finally resealing the break [for reviews, see Gellert (1981) and Wang (1985)]. Topoisomerase I is involved in DNA metabolism and functions during transcription and replication to reduce torsional stress in DNA [reviewed by Wang (1985, 1991)]. Topoisomerase I has been shown to be essential for the growth and development of multicellular organisms such as *Drosophila melanogaster*, whereas the enzyme seems to be nonessential in yeast (Lee et al., 1993). Recently, topoisomerase I was found to be a cofactor for transcription mediated by RNA polymerase II, affecting both basal transcription and activator-dependent transcription (Merino et al., 1993; Kretzschmar et al., 1993). Topoisomerase I also indirectly maintains genome stability due to its role in DNA repair (Wang et al., 1990; Stevnsner & Bohr, 1993; Gangloff et al., 1994). More recently, topoisomerase I was identified as the specific kinase which phosphorylates SR protein[1] splicing factors such as ASF/SF2 that belong to a large family of nuclear phosphoproteins with conserved serine/arginine repeats (Rossi et al., 1996). On the basis of this newly discovered function, we think that topoisomerase I potentially plays a role in RNA splicing.

Topoisomerase I has been identified as the target for several classes of anticancer agents, including camptothecin, actinomycin, intoplicine, and compounds belonging to the fagaronine series (Hsiang et al., 1985; Trask & Muller, 1988; Riou et al., 1993; Larsen et al., 1993). There is general agreement that these agents act by blocking the formation of a transient complex between the enzyme and DNA, i.e., the "cleavable complex" (Chen & Liu, 1994). However, although the formation of this initial complex is readily reversible, additional events might occur which convert it to irreversible lesions, resulting in cell death (Pommier et al., 1994).

The capacity of the cell to choose between DNA repair processes and cell death in response to damaging agents depends on the presence of certain proteins. One of these proteins is the *p53* tumor suppressor gene, which is among the most commonly mutated genes in human tumors and is closely implicated in the regulation of cell proliferation. *p53* is able to bind DNA in a sequence-specific manner (Kern et al., 1991; Funk et al., 1992), to stimulate the annealing of single-stranded DNA (Oberosler et al., 1993; Bakalkin et al., 1994), and to bind many cellular proteins (Pietenpol & Vogelstein, 1993). Overexpression of *p53* can either arrest the cell cycle in the late G1 phase or induce apoptosis, depending on the cell type or the presence of other genetic factors. The growth arrest mediated by *p53* after DNA damage probably allows the cell time to repair DNA prior to replication [for reviews, see Greenblatt et al. (1994), Lane

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[1] Abbreviations: GST, glutathione *S*-transferase; *p53,* tumor suppressor gene or protein *p53*; PCNA, proliferating cell nuclear antigen; SR protein, splicing factor with conserved arginine/serine repeat; SR-kinase, protein kinase that phosphorylates arginine/serine repeats of SR protein; TFIIH, transcription factor IIH; TBP, TATA-binding protein; topoisomerase I, DNA topoisomerase I; DTT, dithiothreitol; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; RPA, replication protein A; XBP, hepatitis B virus X protein.

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(1994), and Levine et al. (1994)].

p53 blocks DNA replication by stimulating the transcription of genes such as $p21^{Waf1/Cip1}$, an inhibitor of cyclin-dependent protein kinases (El-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993), or by directly inhibiting essential replication factors such as RPA or DNA helicase (Wang et al., 1989; Dutta et al., 1993; Oberosler et al., 1993). *p53* indirectly stimulates DNA repair through GADD45, which forms complexes with PCNA, thereby stimulating the DNA synthesis associated with the excision repair process (Kastan et al., 1992; Smith et al., 1994). Moreover, *p53* binds to ERCC3 excision repair factor *in vitro* and to several TFIIH-associated factors (Wang et al., 1994, 1995); thus, *p53* may play a more direct role in DNA repair than heretofore supposed.

The present work is based on the interesting initial observation that exposure to the antitumor agent mitomycin C stimulates nuclear topoisomerase I DNA relaxation activity in MCF7 cells. Mitomycin C is not a topoisomerase inhibitor, but rather induces DNA damage through alkylation (Sartorelli et al., 1994), and is also known to stimulate nuclear accumulation of *p53* (Fritshe et al., 1993). We here show that topoisomerase I coprecipitates with *p53* in nuclear extracts from mitomycin C-treated MCF7 cells and that the increased topoisomerase I activity occurs in parallel with the nuclear accumulation of *p53*. In order to further establish a direct association between topoisomerase I and *p53*, reconstitution experiments were carried out using purified topoisomerase I and *p53*. We find that *p53* binds to topoisomerase I and activates its catalytic DNA relaxation and SR-kinase activities *in vitro*. These results suggest that *p53* might, at least in part, modulate the cellular response to DNA-damaging agents through stimulation of topoisomerase I activities.

EXPERIMENTAL PROCEDURES

Drugs, Chemicals, and Enzymes. Camptothecin and mitomycin C were purchased from Sigma Chemical (La Verpillière, France). Camptothecin was dissolved in dimethyl sulfoxide at 1 mM and then further diluted in water. Mitomycin C was dissolved in PBS at 1 mg/mL and further diluted in cell culture medium.

Cell Treatment and Nuclear Extract Preparation. MCF7 human mammary adenocarcinoma cells were grown in MEM 10% fetal calf serum. About 3×10^6 cells in exponential growth phase were treated for 4 h with or without 10 μg/mL mitomycin C. Cells were washed in PBS and trypsinized. Nuclear extracts were prepared from isolated nuclei in parallel for both treated and control cells as described (Zieve & Penman, 1981; Bach et al., 1990), except that they were dialyzed against 10 mM Tris (pH 7.9), 100 mM KCl, 0.2 mM EDTA, and 0.5 mM DTT after extraction. Nuclear extracts (500 μL) were centrifuged for 4 min at 17000*g* to remove aggregated proteins. The supernatant was mixed with an equal volume of 4 M ammonium sulfate and incubated at 4 °C for 1 h. Bulk proteins were pelleted by centrifugation (10 min at 17000*g*), and 248 mg (3.9 M) of ammonium sulfate was added to the supernatant. After 1 h at 4 °C, the protein precipitate was collected by centrifugation and resuspended in 500 μL of 50 mM Hepes (pH 7.0), 10 mM $MgCl_2$, 3 mM $MnCl_2$, 50 mM KCl, and 0.5 mM DTT.

For the determination of *p53* levels and topoisomerase I activity with respect to the length of treatment, MCF7 cells were harvested at different times (0.5, 2, and 4 h) after the addition of mitomycin C and nuclear extracts prepared as described above.

Immunofluorescence. MCF7 cells were washed in PBS and fixed with 4% formaldehyde for 10 min at 4 °C. Following two washes of 5 min each with PBS, fixed cells were permeabilized with 0.1% Triton X100 for 1 min. After two washes with PBS, fixed cells were incubated with a 1/50 dilution of Pab 1801 anti-*p53* antibody (Oncogene Science) in PBS with 0.5% BSA for 1 h at room temperature. Then they were rinsed twice with PBS and 0.5% BSA and incubated with a 1/100 dilution of biotinylated anti-mouse antibody (Sigma). The presence of *p53* was revealed by using a 1/100 dilution of streptavidin coupled to Texas Red (Amersham) in PBS−BSA and visualized by fluorescence microscopy.

Immunoblot Analysis. Ammonium sulfate precipitates from mitomycin C-treated or control MCF7 cells were subjected to a 4 to 12% gradient sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), and the proteins were transferred to a nitrocellulose filter. Immunoblotting was performed according to the procedure of the ECL kit from Amersham. Anti-topoisomerase I antibody (scl70) was obtained from Topogen (Columbus, OH). The anti-*p53* antibody (Pab 1801) was obtained from Oncogene Science (Uniondale, NY).

Immunoprecipitation of p53. About 3×10^7 cells in exponential growth phase were treated for 4 h with or without 10 μg/mL mitomycin C, and nuclear extract (500 μL) was prepared as described above and adjusted to the same final protein concentration using 10 mM Tris (pH 7.9), 100 mM KCl, 0.2 mM EDTA, and 0.5 mM DTT. Four hundred microliters of each nuclear extract was incubated for 1 h on ice, under gentle agitation, in the presence of 10 μL of monoclonal anti-*p53* antibody (Ab-01, Oncogene Science). One hundred microliters of Protein A-Sepharose (CL-4B, Pharmacia Biotech), prepared in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, and 10 mM 2-mercaptoethanol, was added to the immunoprecipitates which were further incubated on ice for 1 h under gentle agitation. The immunoprecipitates were washed twice with buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 250 mM KCl, 0.1% (w/v) Nonidet P40, 0.02% (w/v) sodium azide, 1 mM PMSF, 5 μg/mL aprotinine, and 1% (w/v) thiodiglycol and once with buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 250 mM KCl, 1 mM PMSF, and 5 μg/mL aprotinine and finally resuspended in 20 μL of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 200 mM KCl, 1 mM PMSF, 1 mM β-mercaptoethanol, and 50% (v/v) glycerol.

Ten microliters of immunoprecipitate was tested for DNA relaxation activity as described later in the text, except that 7.5 mM $MgCl_2$ was added to the reaction mixture. The presence of *p53* was also determined by Western blot, as described above.

p53 Purification. Human *p53* cDNA was cloned into pET11-d expression plasmid (Novagen) and transformed into BL21(DE3) *Escherichia coli* host strain (Novagen) to allow the expression of the whole *p53* protein. Cells were lysed in 20 mM Tris-HCl (pH 8.0), 10% glycerol, 50 mM KCl, 4 mM EDTA, 5% glycerol, 2 mM DTT, 0.5 mM PMSF, and 0.5 mg/mL lysozyme, and then DNA was precipitated by stirring with 0.5 M NaCl and 0.5% polymine P. Proteins in the supernatant were precipitated with 35% (w/v) ammonium

sulfate, and the pellet containing *p53* was collected by centrifugation at 10000*g* for 20 min and dialyzed against H_A buffer [20 mM Tris-HCl (pH 7.5), 200 mM KCl, 0.1 mM EDTA, 5% glycerol, 1 mM dithiotrheitol, and 0.1 mM PMSF]. Dialysate was loaded onto a heparin Sepharose column (Pharmacia), washed with 10 volumes of H_A buffer, and eluted with a linear gradient of 0.2 to 1.0 M KCl in H_A buffer. *p53* eluted between 0.45 and 0.7 M KCl and was concentrated 50-fold on Centricone-10 and then loaded onto a Sephacryl S-300HR gel filtration column equilibrated in H_A buffer without glycerol. *p53* eluted in the high-molecular weight fraction and was concentrated to the loading volume and then dialyzed against storage buffer [20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 2 mM DTT, and 20% glycerol]. After determination of the protein concentration, *p53* was stored in aliquots at −80 °C.

Human *p53* cDNA was cloned between the *Bam*HI and *Eco*RI sites of plasmid pGEX-2T (Pharmacia) to allow the production of a GST fusion protein. GST*p53* fusion protein was produced in *E. coli* TG1 strain and purified on glutathione−agarose (Sigma) as described (Smith & Johnson, 1988).

Topoisomerase I Purification. DNA topoisomerase I from murine P388 or human HeLa and HL60 cells was prepared according to Rossi et al. (1996), starting from 2×10^7 cells in the exponential growth phase. Nuclear extracts and ammonium sulfate precipitates were prepared as described above for MCF7 cells. The ammonium sulfate pellet was resuspended in 500 μL of buffer A consisting of 50 mM Hepes (pH 7.0), 10 mM $MgCl_2$, 3 mM $MnCl_2$, 50 mM KCl, and 0.5 mM DTT and incubated for 30 min at 4 °C with 50 μL of nickel agarose beads (probond resin, Invitrogen) equilibrated in the same buffer. After two washes with 300 μL of buffer, bound topoisomerase I was eluted with 300 μL of buffer A containing 40 mM imidazole as a single 100 kDa polypeptide band.

Topoisomerase I was also purified from 200 g of frozen calf thymus glands by two successive columns of phosphocellulose and hydroxyapatite as described previously (Riou et al., 1986).

DNA Relaxation Assay. Topoisomerase I and *p53* at different concentrations (as specified in the text) were mixed on ice in a final volume of 20 μl of 20 mM Tris-HCl (pH 7.5), 150 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, and 0.5 μg of pBR322 DNA. The reaction mixtures were incubated at 37 °C for 30 min and the reactions stopped on ice with 6 μL of loading buffer containing 50 mM EDTA, 0.5% SDS, 0.1% bromophenol blue, and 50% (w/v) sucrose. The samples were submitted to electrophoresis in 1% agarose gels at 2 V/cm for 12−18 h in TBE buffer [30 mM Tris base, 90 mM boric acid, and 2 mM EDTA (pH 8.0)]. Gels were stained with ethidium bromide and photographed under UV light.

DNA Cleavage Assay. ^{32}P-end-labeled pBR322 DNA was prepared as described previously (Riou et al., 1991). Topoisomerase I at different concentrations (see text) was mixed on ice with 300 ng of GST*p53* in a final volume of 20 μL of 20 mM Tris-HCl (pH 7.5), 60 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, and 20 000 dpm of 3′ ^{32}P-end-labeled pBR322 DNA. Samples were incubated at 37 °C for 10 min, and the reactions were terminated by the addition of 2 μL of 2.5% SDS and 2.5 mg/mL proteinase K. Samples were further incubated for a period of 30 min at 50 °C, and DNA was denatured by the addition of 10 μL of 0.45 M NaOH, 30 mM EDTA, 15% (w/v) sucrose, and 0.1% bromocresol green. Samples were loaded on a 1% agarose gel in TBE with 0.1% SDS. After electrophoresis overnight at 2 V/cm, gels were dried and autoradiographed for 1 day (Hyperfilms, Amersham).

SR-Kinase Assay. Purified recombinant ASF/SF2 protein was prepared according to Ge et al. (1991). This protein is a specific substrate of the topoisomerase I kinase reaction (Rossi et al., 1996). The reaction mixtures contained 300 ng of ASF/SF2 protein in 50 mM Hepes (pH 7.0), 10 mM $MgCl_2$, 3 mM $MnCl_2$, 50 mM KCl, 0.5 mM DTT, 3 μCi [γ-^{32}P]ATP (300 Ci/mmol), and the indicated amounts of MCF7 ammonium sulfate precipitates or purified HeLa topoisomerase I (10 ng) in a final volume of 10 μL. When purified topoisomerase I was used, GST*p53* or GST was added to the reaction mixture (see text). Samples were incubated at 30 °C for 30 min, mixed with 5 μL of (3×) Laemmli loading buffer, and applied to a 10% SDS−polyacrylamide gel. Labeled ASF/SF2 was revealed by autoradiography.

Sucrose Density Gradient Analysis. One hundred microliters of calf thymus topoisomerase I (30 μg) with or without GST*p53* (3 μg) in 20 mM Tris-HCl (pH 7.5), 150 mM KCl, 0.5 mM EDTA, and 0.5 mM DTT was incubated for 15 min at 37 °C and cooled on ice. Each sample was deposited on the top of a 5 to 20% sucrose gradient (4 mL) prepared in the same buffer and ultracentrifuged in a Beckman SW55 rotor for 6 h at 50 000 rpm at 4 °C. Fractions of 200 μL were collected from the bottom, and an aliquot of each (2 μL) was tested for DNA relaxation activity as described above. Results presented were representative of three independent experiments.

Heat Denaturation Assay. Purified calf thymus topoisomerase I (10, 5, and 2.5 ng) was mixed on ice with or without 300 ng of *p53* in a final reaction volume of 18 μl containing 20 mM Tris-HCl (pH 7.5), 150 mM KCl, 0.5 mM EDTA, and 0.5 mM DTT. Samples were incubated for 40 min either on ice or at 45 °C. Two microliters of pBR322 DNA (0.5 μg) was added to each tube, and samples were further incubated for 30 min at 37 °C. Alternatively, topoisomerase I (10, 5, and 2.5 ng) was mixed directly with 0.5 μg of pBR322 DNA in the same conditions and incubated for 40 min at 45 °C. Two microliters of *p53* (300 ng) was added and the incubation continued for 30 min at 37 °C. Reactions were stopped and analyzed by agarose gel electrophoresis as described above. Photographic negatives of the gel pictures were scanned with a Pharmacia ultroscan densitometer in order to quantify the relaxation reaction.

RESULTS

Treatment of MCF7 Cells with Mitomycin C Stimulates Topoisomerase I Activities and the Nuclear Accumulation of p53. In order to determine the influence of *p53* on topoisomerase I activities, MCF7 cells were treated with mitomycin C (10 μg/mL for 4 h), an antitumor agent that induces the nuclear accumulation of *p53* (Fritsche et al., 1993). To confirm that *p53* was translocated to the nucleus under our experimental conditions, cells were stained with an anti-*p53* antibody and examined by immunofluorescence microscopy. As shown in Figure 1, untreated MCF7 cells (panel A) presented a disperse background cytoplasmic

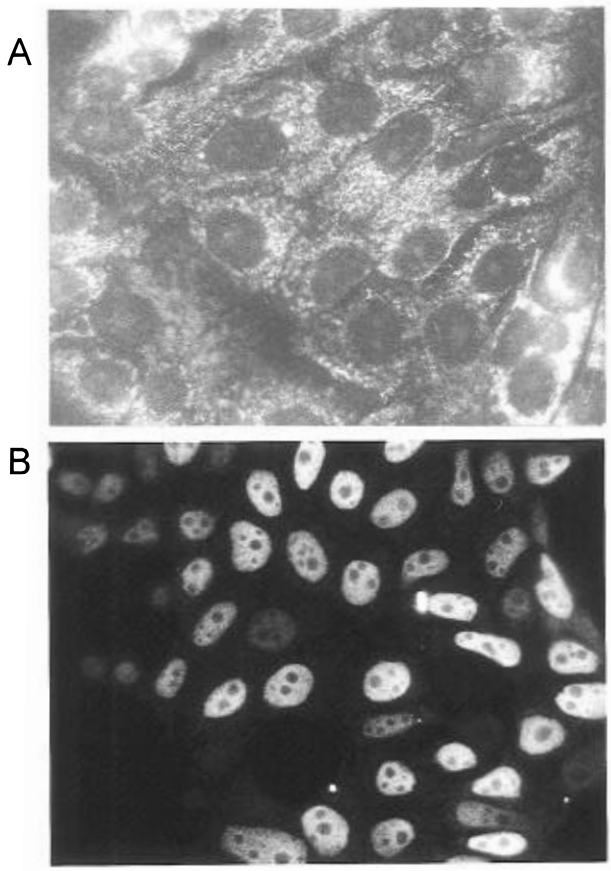

FIGURE 1: Detection of *p53* by immunofluorescence in MCF7 cells either untreated (A) or treated with mitomycin C (10 μg/mL for 4 h) (B). Low and diffuse cytoplasmic fluorescence was detected in untreated cells, whereas the staining was intense in the nuclei of MCF7 cells treated with mitomycin C.

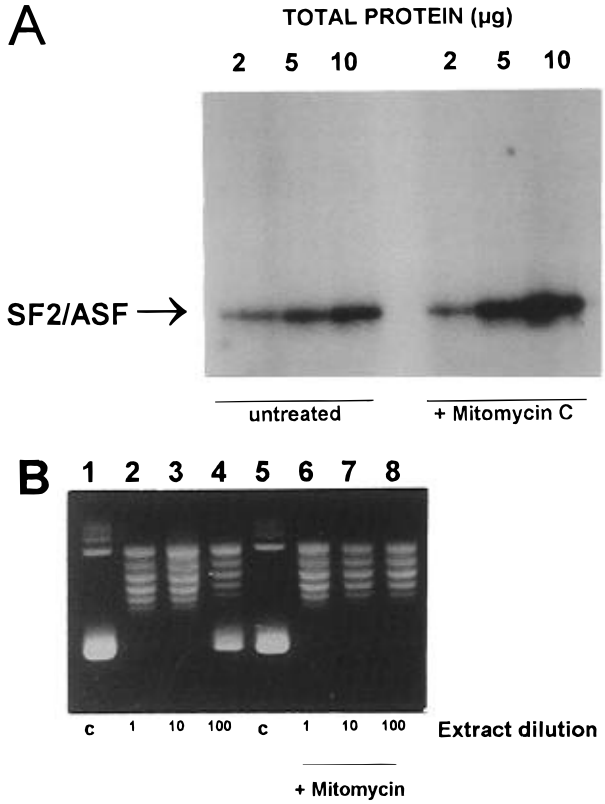

FIGURE 2: Ammonium sulfate precipitates (750 μg/mL protein) recovered from nuclei of control or mitomycin C-treated MCF7 cells were assayed for (A) topoisomerase I protein kinase activity with recombinant ASF/SF2 protein as substrate in the presence of [γ-^{32}P]ATP and (B) topoisomerase I-catalyzed DNA relaxation of supercoiled pBR322 DNA. Control supercoiled DNA (lanes 1 and 5) was incubated with 1-, 10-, and 100-fold dilutions of extracts from control cells (lanes 2, 3, and 4) or with 1-, 10-, and 100-fold dilutions of extracts from mitomycin C-treated cells (lanes 6, 7, and 8).

fluorescence without any noticeable intensity within the nucleus. In contrast, MCF7 cells treated by mitomycin C (panel B) had a strong nuclear fluorescence, characteristic of *p53* translocation.

In parallel experiments, nuclei were prepared immediately after drug treatment and nuclear proteins were extracted with high salt buffer containing 50 mM $MgCl_2$. Topoisomerase I was concentrated by two ammonium sulfate precipitations. More than 95% of the initial topoisomerase I activity was recovered in the second ammonium sulfate precipitate. The catalytic activities of topoisomerase I in the precipitates were also evaluated by measuring the kinase activity on recombinant ASF/SF2 SR protein (SR-kinase assay, Figure 2A) and the relaxation of supercoiled pBR322 DNA (relaxation assay, Figure 2B). As shown in Figure 2A, SDS−PAGE analysis of the phosphorylated products indicated a greater SR-kinase activity in the precipitates from treated MCF7 cells than in those from untreated cells. Quantification of the phosphorylated products by densitometry revealed that SR-kinase activity was stimulated by 2.4-fold in mitomycin C-treated MCF7 cells. Similar results were found with the topoisomerase I relaxation assay (Figure 2B), since the fraction of relaxed DNA was greater in the mitomycin C-treated preparation than in the untreated preparation, when identical amounts of total proteins were assayed (compare lanes 4 and 8). Quantification by more precise serial dilutions (not shown) indicated that topoisomerase I relaxation activity was stimulated 13-fold in mitomycin C-treated MCF7 cells compared to that of untreated controls.

Therefore, the topoisomerase I activity was higher in preparations from mitomycin-treated MCF7 cells than in those from untreated cells as determined by two independent assay systems.

Increased p53, but Not Topoisomerase I, Protein Levels in Mitomycin C-Treated MCF7 Cells. Immunoblot analysis with anti-*p53* antibodies revealed that purified nuclear extracts from MCF7 cells treated by mitomycin C were enriched in *p53* compared to extracts from untreated cells (Figure 3A). Immunoblot analysis with anti-topoisomerase I antibody (Figure 3B) also indicated that the amount of topoisomerase I was detected at comparable levels, since topoisomerase I from treated cells represented 70% of that from untreated cells, as determined by densitometric scanning.

These results, together with the observation that insect topoisomerase I activity was present in samples of human *p53* purified from baculoviruses (J.-F. Riou, unpublished results), suggested that *p53* forms molecular complexes with topoisomerase I. The relationship between topoisomerase I activation and the nuclear accumulation of *p53* in mitomycin C-treated cells was then determined. Cells were harvested at different times after drug treatment, and topoisomerase I DNA relaxation activity and *p53* protein levels were measured in nuclear extracts. The results which are presented in Figure 4 indicate that the topoisomerase I activity was stimulated within 30 min, with progressive increase up

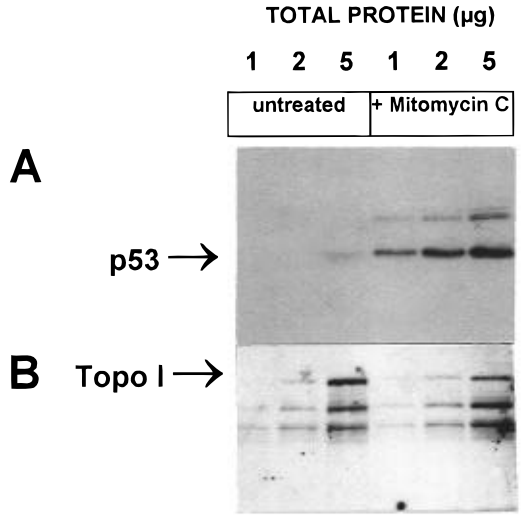

FIGURE 3: Topoisomerase I and *p53* proteins in nuclear extracts from untreated or mitomycin C-treated MCF7 cells. The indicated amounts of ammonium sulfate-precipitated proteins were loaded, submitted to electrophoresis in a 4 to 12% gradient SDS−PAGE gel, and transferred to nitrocellulose. (A) Immunoblot probed with antibodies directed toward mouse *p53*. (B) Immunoblot probed with antibodies directed toward human topoisomerase I. The position of 100 kDa topoisomerase I is indicated by an arrow. The other bands correspond to artifactual cross-reactions of anti-human IgG and are also detected with the secondary antibody alone.

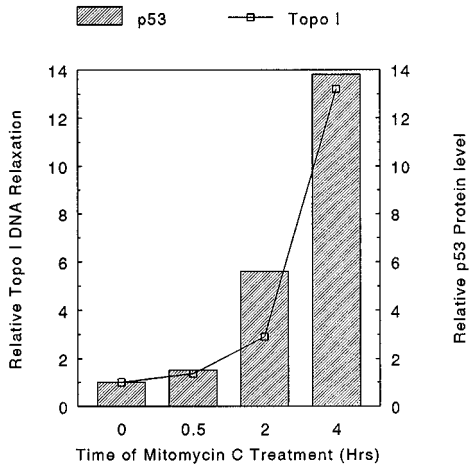

FIGURE 4: Relationship between topoisomerase I activation and the nuclear accumulation of *p53* in mitomycin C-treated MCF7 cells. Cells were harvested at different times after drug treatment, and nuclear extract was prepared; topoisomerase I DNA relaxation activity was determined by serial dilution, and *p53* protein levels were determined by Western blotting. Photograpic negatives of the DNA relaxation assay and an autoradiograph of the Western blot were scanned in order to quantify the assays. The results were expressed relative to the values obtained in untreated cells (time = 0), which was arbitrarily defined as equal to 1.

to 4 h. A similar increase in protein levels was also observed for *p53*. Therefore, these results indicate that the topoisomerase I activation parallels the nuclear accumulation of *p53*, which is consistent with the idea that the stimulation of topoisomerase I activity is due to association with *p53*.

Effect of Recombinant p53 on DNA Relaxation Activity from Purified Mammalian Topoisomerase I. To answer this question, reconstitution experiments with purified *p53* and topoisomerase I were carried out. The effect of recombinant *p53* or GST*p53* fusion protein on the catalytic activity of purified topoisomerase I was determined by DNA relaxation assays. As shown in Figure 5A, *p53* stimulated the

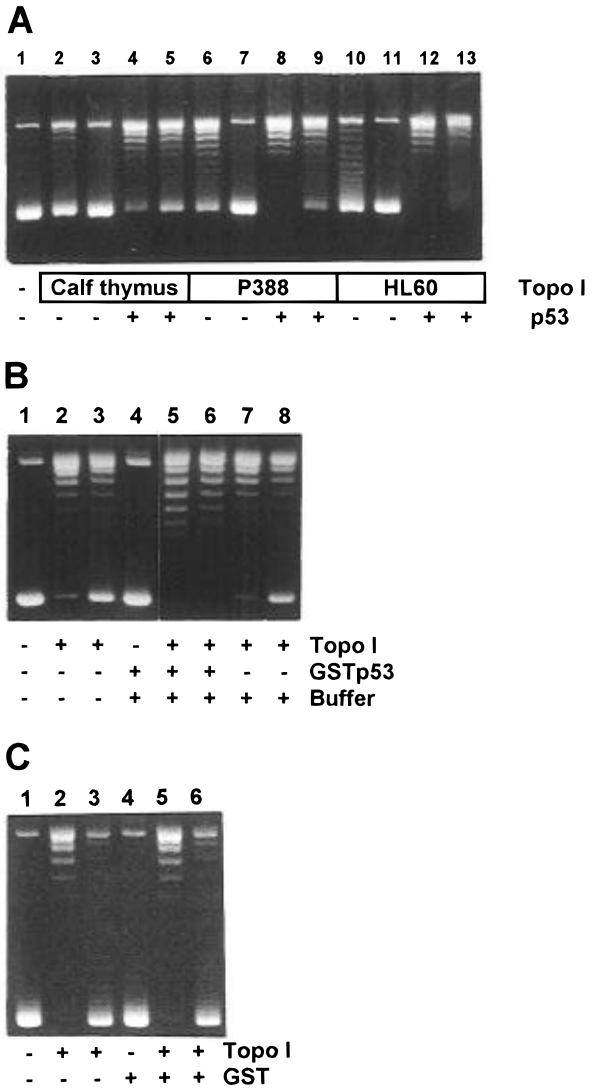

FIGURE 5: Stimulation of topoisomerase I DNA relaxation activity by *p53* or GST*p53*. (A) Effect of *p53*. Topoisomerase I from calf thymus, P388 murine leukemia cells, or HL60 human leukemia cells at 10 ng (lanes 2, 4, 6, 8, 10, and 12) or 2 ng (lanes 3, 5, 7, 9, 11, and 13) was incubated with 300 ng of *p53* as indicated on the figure. (B) Effect of GST*p53*. Calf thymus topoisomerase I at 20 ng (lanes 2, 5, and 7) or 10 ng (lanes 3, 6, and 8) was incubated alone (lanes 2 and 3) or with 300 ng of GST*p53* (lanes 5 and 6) or with GST*p53* dilution buffer (lanes 7 and 8). Lane 4 is GST*p53* without topoisomerase I. (C) GST itself has no effect; calf thymus topoisomerase I at 20 ng (lanes 2 and 5) or 5 ng (lanes 3 and 6) was incubated alone (lanes 2 and 3) or with 300 ng of GST (lanes 5 and 6). Lane 4 is GST (300 ng) without topoisomerase I. For panels A−C, lane 1 is the control supercoiled pBR322 DNA.

relaxation of supercoiled DNA by topoisomerase I isolated from calf thymus (lanes 4 and 5), P388 (lanes 8 and 9), and HL60 (lanes 12 and 13) at enzyme concentrations (0.2 unit) where less than 10% of the DNA was relaxed in the absence of *p53*.

Stimulation of topoisomerase I-mediated DNA relaxation was also observed for GST*p53* (Figure 5B, lanes 5 and 6). Control reactions performed with DNA and GST*p53* (lane 4) or with topoisomerase I and GST dilution buffer (lanes 7 and 8) had no effects. In addition, GST alone at the same concentration (300 ng) as GST*p53* had no effect on the topoisomerase I reaction (Figure 5C, compare lanes 2 and 3 with lanes 5 and 6).

Increasing amounts of *p53* (75−1200 ng) incubated with a fixed concentration (10 ng) of topoisomerase I induced a

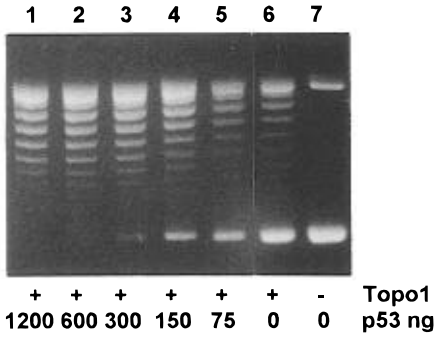

FIGURE 6: Topoisomerase I relaxation activity in the presence of different concentrations of *p53*. Ten nanograms of calf thymus topoisomerase I was incubated with 1200, 600, 300, 150, 75, and 0 ng of *p53* (lanes 1−6); lane 7 is control pBR322 DNA.

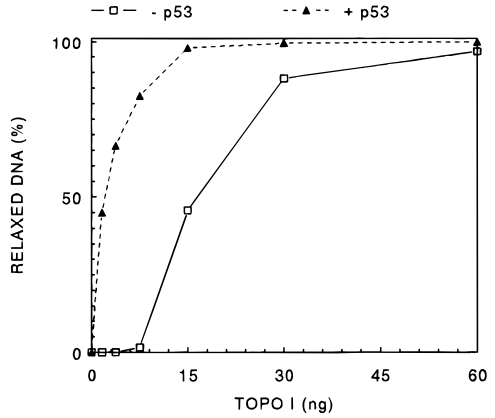

FIGURE 7: Effect of GST*p53* (300 ng) on different concentrations of calf thymus topoisomerase I. After 30 min of incubation at 37 °C, DNA samples were analyzed by agarose gel electrophoresis and DNA relaxation was quantified by densitometric scanning: control topoisomerase I (□) and topoisomerase I with GST*p53* (▲).

dose-dependent stimulation of the relaxation reaction (Figure 6). Stimulation of DNA relaxation was detectable with 75 ng of *p53* (lane 5), and complete DNA relaxation was achieved with 300 ng of *p53* (lane 3). Increasing the *p53* concentration to 600 and 1200 ng gave the same maximal relaxation reaction (lanes 1 and 2) as observed with 300 ng. We also incubated increasing amounts of topoisomerase I (1.8−60 ng) with a fixed concentration of *p53*, as shown in Figure 7. The results indicate that the same degree of DNA relaxation was achieved with 15 ng of topoisomerase I in the absence of *p53* as with 1.8 ng of topoisomerase I in the presence of 300 ng of *p53*.

p53 Stimulates the Site-Specific DNA Cleavage Activity Mediated by Calf Thymus Topoisomerase I. The effect of *p53* on the site-specific DNA cleavage mediated by topoisomerase I was also examined. In this assay, topoisomerase I cleavage sites in pBR322 DNA were mapped by using the antitumor drug camptothecin, which inhibits the religation step of topoisomerase I catalysis, thereby causing the accumulation of normally short-lived cleavage intermediates. The resulting covalent topoisomerase I−DNA complexes mask a single-stranded DNA break which can be revealed by proteolysis and DNA strand separation. As shown in Figure 8, topoisomerase I (6−0.12 μg, lanes 2−5) induced dose-dependent DNA cleavage, as indicated by a decrease in the full-length pBR322 DNA band and the appearance of

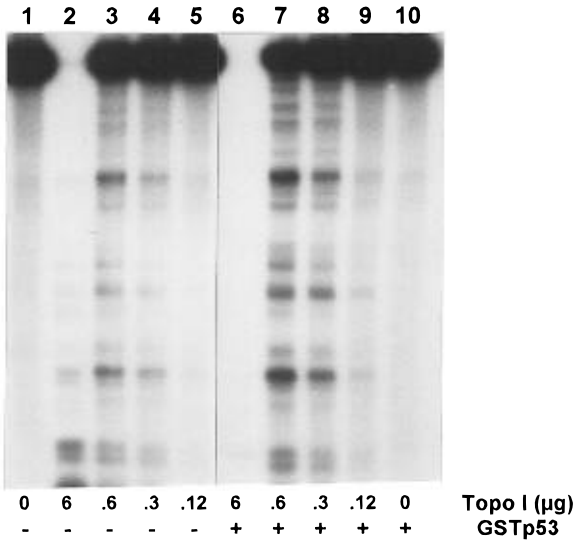

FIGURE 8: Effect of GST*p53* on the topoisomerase I-mediated DNA cleavage reaction. Calf thymus topoisomerase I (6−0.12 μg) was incubated in the absence (lanes 2−5) or presence of 300 ng of GST*p53* (lanes 6−9) with linear 3′ α-^{32}P-labeled pBR322 DNA as indicated in Experimental Procedures. DNA cleavage was revealed by autoradiography after agarose gel electrophoresis. Lane 1 contains control DNA and lane 10 DNA incubated with GST*p53* in the absence of topoisomerase I.

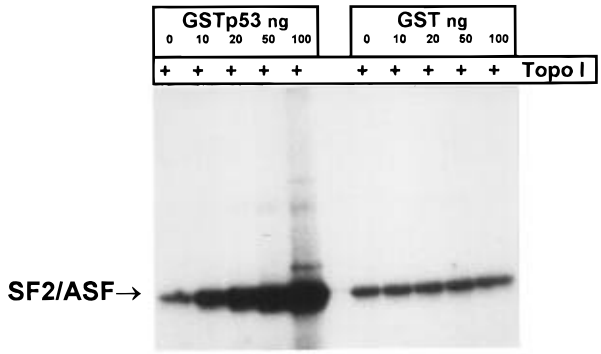

FIGURE 9: Effect of GST*p53* and GST on the SR kinase activity of topoisomerase I. Purified topoisomerase I (10 ng), ASF/SF2 (300 ng), and [γ-^{32}P]ATP were incubated with or without GST*p53* or GST (10−100 ng) at increasing concentrations, as described in Experimental Procedures, and then electrophoresed in 10% polyacrylamide gels. Phosphorylated ASF/SF2 was revealed by autoradiography.

specific DNA cleavage bands. In the presence of 300 ng of GST*p53* (lanes 6−10), a significant increase in topoisomerase I cleavage was observed for all concentrations of topoisomerase I tested, i.e., cleavage of DNA fragments in smaller pieces in lane 6, increased cleavage of full-length DNA, and stimulation of major cleavage sites in lanes 7−9. No new topoisomerase I cleavage sites were induced by the inclusion of *p53* in the cleavage reactions. These data indicate that the interaction of *p53* with topoisomerase I results in the formation of more intermediate reaction products; this may explain how *p53* stimulates the relaxation reaction.

p53 Stimulates the SR-Kinase Activity of Topoisomerase I. We also examined the effect of GST*p53* on the SR-kinase activity of purified topoisomerase I. As shown in Figure 9, GST*p53* induced a significant increase in the topoisomerase I-mediated phosphorylation of SF2/ASF. The effect of *p53* was dose-dependent and was already apparent at a nearly equimolar ratio of *p53* and topoisomerase I (10 ng). In a

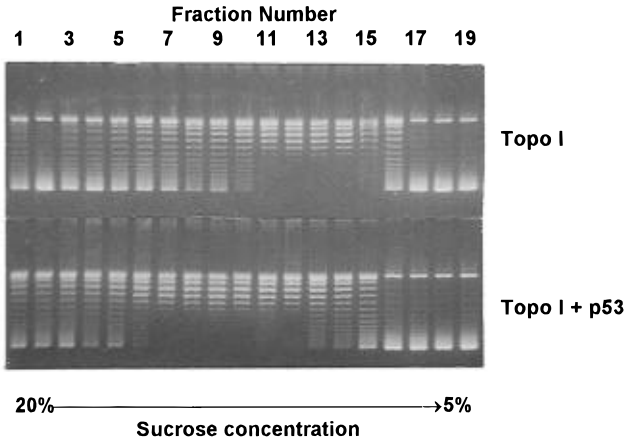

FIGURE 10: Sucrose gradient sedimentation of topoisomerase I in the presence or absence of *p53*. Fractions of 200 μL were collected from the bottom, and a 2 μL aliquot of each was tested for DNA relaxation activity. In the presence of *p53*, one part of the topoisomerase I activity sedimented at higher sucrose concentrations.

control experiment with purified GST, GST by itself had no effect on the topoisomerase I-mediated kinase reaction even at a 30-fold molar excess of GST (100 ng) over topoisomerase I. This result indicates that the DNA binding property of *p53* is not a prerequisite for the stimulation of topoisomerase I but appears to be due to a direct interaction between the two proteins.

p53 and Topoisomerase I Are Physically Associated in the Absence of DNA. In order to determine whether topoisomerase I and *p53* form molecular complexes, topoisomerase I was subjected to sucrose density gradient analysis in the presence or absence of *p53*. The experiment was done in the absence of DNA and under conditions where topoisomerase I-induced DNA cleavage and SR-kinase activities, but not DNA relaxation activity, were stimulated, i.e., with *p53* and topoisomerase I at an equimolar ratio. A 2 μL aliquot of each fraction was assayed for DNA relaxation activity, as shown in Figure 10. Under our experimental conditions, maximal topoisomerase I activity was detected in fractions 11−14. When topoisomerase I was preincubated with *p53*, DNA relaxation activity was found in fractions 7−12. These results indicate that some of the topoisomerase I sedimented at higher sucrose concentrations in the presence of *p53*, which strongly suggests that *p53* binds directly to topoisomerase I in the absence of DNA.

On the basis of these results, we assumed that the physical association between *p53* and topoisomerase I might modify the thermal tolerance of topoisomerase I, which has been reported to be rapidly inactivated by short heat treatments at 45 °C (Ciavarra et al., 1994). By contrast, topoisomerase I was stable by incubation on ice without DNA for at least 1 h (not shown). As shown in Figure 11, calf thymus topoisomerase I incubated in ice for 40 min at a concentration giving partial DNA relaxation was fully activated in the presence of *p53*. Calf thymus topoisomerase I treated for 40 min at 45 °C in the absence of *p53* completely lost its catalytic activity. The addition of *p53* (300 ng) markedly increased the thermal stability of topoisomerase I, since about 50% of the initial relaxation activity was retained. In contrast, the addition of *p53* after the thermal inactivation of topoisomerase I could not reactivate the enzyme activity (results not shown). These findings are consistent with the

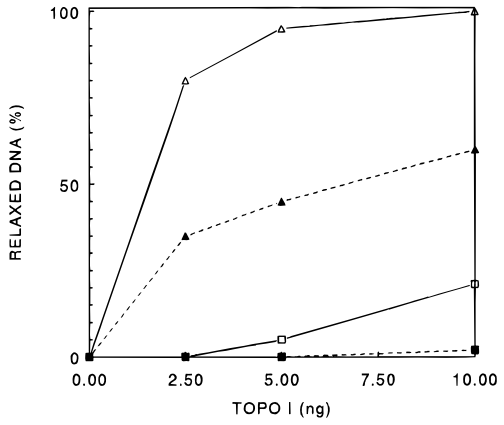

FIGURE 11: *p53* protects topoisomerase I from thermal inactivation *in vitro*. Calf thymus topoisomerase I (10, 5, and 2.5 μg) was incubated for 40 min in the reaction buffer in the absence or presence of *p53* (300 ng), either in ice or at 45 °C. DNA (0.5 μg) was then added, and all samples were incubated for 30 min at 37 °C for determination of the topoisomerase I-catalyzed relaxation of DNA: topoisomerase I at 0 °C (□), topoisomerase I + *p53* at 0 °C (△), topoisomerase I at 45 °C (■), topoisomerase I + *p53* at 45 °C (▲).

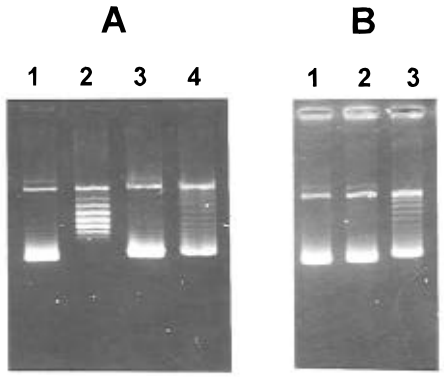

FIGURE 12: *p53* and topoisomerase I activity associate *in vivo*. DNA relaxation assay was determined with *p53*-immunoprecipitated proteins from MCF7 nuclear extracts prepared in parallel for both mitomycin C-treated and untreated control cells. Panels A and B corresponded to two independent experiments: (A) lane 1, control pBR322 DNA; lane 2, DNA incubated with purified calf thymus topoisomerase I; lane 3, DNA incubated with 10 μL of immunoprecipitate from untreated control cells; lane 4, DNA incubated with 10 μL of immunoprecipitate from treated cells; (B) lane 1, control pBR322 DNA; lane 2, DNA incubated with 10 μL of immunoprecipitate from untreated control cells; lane 3, DNA incubated with 10 μL of immunoprecipitate from treated cells.

formation of molecular complexes between *p53* and topoisomerase I *in vitro*.

p53 and Topoisomerase I Associate in Vivo. In order to determine whether *p53* and topoisomerase I were able to associate *in vivo*, nuclear extracts prepared from mitomycin C-treated and untreated MCF7 cells were immunoprecipitated by anti-*p53* antibody and tested for the presence of topoisomerase I DNA relaxation activity. Two independent experiments are presented in Figure 12A,B. The results clearly indicate that ATP-independent DNA relaxation activity was present in the *p53* immunoprecipitates recovered from mitomycin C-treated MCF7 cells (Figure 12A, lane 4, and Figure 12B, lane 3) but absent in the immunoprecipitates recovered from untreated cells (Figure 12A, lane 3, and Figure 12B, lane 2). These results support the formation of molecular complexes between the two proteins *in vivo* after treatment with mitomycin C.

DISCUSSION

We have studied the variations of topoisomerase I levels and activity in MCF7 cells under conditions where *p53* expression was induced by DNA-alkylating agent mitomycin C (Fritsche et al., 1993). No significant changes of topoisomerase I protein levels were observed when *p53* was induced, in agreement with the results of Canman et al. (1994). However, the catalytic activity of topoisomerase I, as measured by relaxation of supercoiled DNA, was increased following *p53* induction. Similar results were observed when NIH 3T3 cells were treated for 3 h with actinomycin D (result not shown), suggesting that the observed effect was not restricted to a unique cellular model nor to a single DNA-damaging agent.

Topoisomerase I was recently identified as the specific kinase responsible for phosphorylation of SR protein splicing factors (Rossi et al., 1996). Our results show that nuclear extracts from mitomycin C-treated MCF7 cells also have an increased protein kinase activity, as measured by phosphorylation of ASF/SF2, compared to untreated control cells. Although ASF/SF2 is a highly specific substrate for topoisomerase I *in vitro* (Rossi et al., 1996), we cannot exclude that the increased kinase activity may be due to a kinase other than topoisomerase I. However, this seems unlikely since *p53* has been reported to induce the expression of kinase inhibitors, such as $p21^{Waf1/Cip1}$, rather than that of kinase activators (El-Deiry et al., 1993; Harper et al., 1993).

Interestingly, immunoblots of partially purified extracts from treated cells, which were enriched in topoisomerase I, also contained *p53*, and time course experiments indicated that drug treatment induced a progressive and parallel increase of both topoisomerase I activity and the levels of nuclear *p53*. These data, together with the observation that insect topoisomerase I activity was present in samples of human *p53* purified from baculovirus, suggested that the two proteins associate in molecular complexes. To determine whether *p53* has a direct effect on topoisomerase I, reconstitution experiments were carried out with purified enzymes.

Human recombinant *p53* and GST*p53* fusion proteins both stimulated the DNA relaxation activity of topoisomerase I isolated from several mammalian sources, whereas the GST protein by itself failed to do so. In contrast, *p53* had no effect on bacterial topoisomerase I isolated from *E. coli* (results not shown).

The effect of *p53* on the different steps of the catalytic cycle of topoisomerase I was determined. The results show that GST*p53* stimulates the formation of covalent DNA−protein complexes in the presence of camptothecin. Furthermore, the religation step, during which the single-stranded DNA break is resealed, is also stimulated by *p53* (results not shown). Therefore, at least two steps in the catalytic cycle of topoisomerase I are stimulated by *p53*.

GST*p53* increased the phosphorylation of the ASF/SF2 splicing factor by purified topoisomerase I *in vitro*. Although the discovery of this kinase activity is recent and little is known about which part of the topoisomerase I molecule is needed for this activity, both SR-kinase and DNA relaxation activities were found to be inhibited by camptothecin derivatives (Rossi et al., 1996). This is consistent with the pleiotropic effect of *p53* on both catalytic activities of topoisomerase I. Since SR-kinase activity was stimulated in the absence of DNA, we supposed that the DNA binding properties of *p53* are not a prerequisite for the activation of topoisomerase I. However, the presence of DNA might influence the way *p53* interacts with topoisomerase I. Whereas the SR-kinase activation was detectable at nearly equimolar concentrations of the two proteins, DNA relaxation was only stimulated by using a 10-fold molar excess of *p53* over topoisomerase I. In contrast, the DNA cleavage reaction, which was carried out with about 25-fold less DNA than the relaxation reaction (and with linear DNA), was stimulated at an equimolar ratio of *p53* and topoisomerase I. Further experiments are needed to elucidate in more detail the role of nucleic acids in the interaction between *p53* and topoisomerase I.

Previous reports indicate that *p53* can bind single-stranded DNA and catalyze DNA renaturation and strand transfer (Oberosler et al., 1993; Bakalkin et al., 1994). In addition, *p53* has been found to inhibit the DNA helicase activity of the SV40 large T antigen and the binding of the DNA replication factor RPA to single-stranded DNA (Wang et al., 1989; Dutta et al., 1993). In contrast to these inhibitory properties of *p53*, our results provide the first evidence that *p53* can also activate at least one enzyme, topoisomerase I, involved in DNA replication and transcription.

p53 has been reported to bind a large number of cellular or viral proteins (Seto et al., 1992; Filhol et al., 1992; Pietenpol et Vogelstein, 1993). Interestingly, some of these proteins, i.e., casein kinase II, Hsc 70, and TBP, also form complexes with topoisomerase I (Merino et al., 1993; Ciavarra et al., 1994; Kordiyak et al., 1994). Furthermore, the large T antigen of SV40 has been found to possess either intrinsic or copurifying topoisomerase I activity (Mann, 1993; Marton et al., 1993). When HeLa cell extracts were run through GST*p53* columns, as recently reported by Sakurai et al. (1994), GST*p53* retained four specific bands, including a band of 100 kDa which corresponds to the molecular mass of topoisomerase I. These data, together with the results we obtained from sucrose gradient analysis and by testing for thermal inactivation of topoisomerase I in the presence of *p53*, strongly suggest that the two proteins are able to form molecular complexes.

Our *in vitro* experiments indicate that the activation of topoisomerase I activities in nuclear extracts from mitomycin C-treated MCF7 cells could be due to association with *p53* but do not exclude the possibility that topoisomerase I is otherwise post-transcriptionally modified. In an attempt to demonstrate the *in vivo* association between *p53* and topoisomerase I, we immunoprecipitated *p53* from nuclear extracts prepared from either mitomycin C-treated or untreated control cells. The results clearly demonstrate that topoisomerase I activity was present only in immunoprecipitates of *p53* from treated cells. These results support an *in vivo* association between topoisomerase I and *p53*.

Since topoisomerase I is present in the nucleus during all the phases of the cell cycle and in both replicating and nonreplicating cells, whereas wild type *p53* usually is absent, our results suggest that the activation of topoisomerase I in mitomycin C-treated MCF7 cells is not likely to be a general physiological phenomenon but restricted to situations where we have DNA damage. Recent evidence indicates that *p53* modulates the TFIIH-associated nucleotide excision repair activity through *in vivo* binding to XBP which leads to inhibition of its helicase activity (Wang et al., 1995). Stimulation of topoisomerase I and its associated DNA

cleavage and religation activities or protein kinase activity by *p53* might represent a way for *p53* to modulate DNA processes downstream from the site of DNA damage such as DNA replication, DNA transcription, and RNA splicing linked to the DNA repair pathway.

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