Replication of damaged DNA *in vitro* is blocked by p53

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ABSTRACT

The tumor suppressor protein p53 may have other roles and functions in addition to its well-documented ability to serve as a sequence-specific transcriptional activator in response to DNA damage. We showed previously that p53 can block the replication of polyomavirus origin-containing DNA (Py ori-DNA) *in vitro* when p53 binding sites are present on the late side of the Py ori. Here we have both further extended these observations and have also examined whether p53 might be able to bind directly to and inhibit the replication of damaged DNA. We found that p53 strongly inhibits replication of γ-irradiated Py ori-DNA and such inhibition requires both the central DNA binding domain and the extreme C-terminus of the p53 protein. An endogenous p53 binding site lies within the Py origin and is required for the ability of p53 to block initiation of replication from γ-irradiated Py ori-DNA, suggesting the possibility of DNA looping caused by p53 binding both non-specifically to sites of DNA damage and specifically to the endogenous site in the polyomavirus origin. Our results thus suggest the possibility that under some circumstances p53 might serve as a direct regulator of DNA replication and suggest as well an additional function for cooperation between its two autonomous DNA binding domains.

INTRODUCTION

The p53 tumor suppressor protein accumulates in the nuclei of cells in response to various forms of stress including DNA damage. Once stabilized, p53 can arrest cells in G_1 and/or G_2 or cause apoptosis, through its functions as a sequence-specific transcriptional regulator (for reviews, see 1–3). The p53 protein possesses structural domains that are characteristic of transcriptional activators including an activation region at its N-terminus, a sequence-specific DNA binding region within its central portion and an oligomerization domain within its C-terminal region. In addition to genes regulating cell cycle and cell death, p53 can stimulate the transcription of genes encoding products that can affect both DNA synthesis and repair. For example, one of the most well studied p53 target genes is p21/CIP1/WAF1, which can both inhibit cyclin-dependent kinases (4) and also bind to and inactivate PCNA, which plays an essential role in DNA replication by facilitating processive DNA synthesis by polymerase δ (5). Moreover, p53 activates both the RRM2B/p53R2 gene (6) and the DDB2 (damage-specific DNA binding protein 2) gene (7,8) that each plays distinct roles in DNA repair.

Although as mentioned above p53 protein embodies the classical features of a sequence-specific transcriptional activator, it has one property that is rather unique, which is that it possesses a second essentially autonomous DNA binding region at its extreme C-terminus (9). This region of the protein has been shown to facilitate and be required for the interaction of p53 with various forms of damaged or altered DNA including deletion/insertion mismatches, DNA ends, as well as double- and single-strand breaks (for a review, see 10). The function of this region is not fully clarified and it has also been shown to be able to regulate the mode by which the central region recognizes DNA (for a review, see 11).

Although p53 is clearly involved in regulating the transcription of numerous targets, there are several lines of evidence suggesting that p53 may play transcription-independent roles in cells as well. Some mutant p53 proteins that have lost transcriptional transactivation capacity retain their ability to arrest cell growth to varying extents (12–16). Conversely, loss of growth suppressor function has been reported in some p53 mutants, which retain transcriptional activity (17–19). A recent study by Jaks *et al.* (20) showed that when cells are treated with the DNA-damaging drug camptothecin, p53 accumulation rather than the transactivation of p21 is required for the G_1 arrest, strongly suggesting that p53 may play transcription-independent role(s) in cells. In fact it is possible that under some conditions p53 may play a direct role in regulating DNA synthesis. Various experimental results suggest that p53 protein may inhibit DNA replication in a transcription-independent manner. The C-terminally truncated form of p53 protein, p53ΔC30, which is also the DNA binding active form of p53 protein, was reported to inhibit *Xenopus* nuclear DNA replication in a transcription-independent manner (21). p53 may inhibit the initiation of DNA replication directly by binding to RP-A (22–24), which is a single-stranded DNA binding protein required for DNA replication. p53 protein also blocks the replication of SV40

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DNA *in vitro* by binding to and inactivating the SV40 large T antigen (25–27). Additionally, although p53 protein does not bind to or inhibit polyoma large T antigen, it is also able to inhibit the replication of polyomavirus origin-containing DNA (Py ori-DNA) *in vitro* although in this case it is necessary for p53-specific binding sites to be present in the template origin-containing plasmid (28).

There have also been reports supporting the possibility of role(s) for p53 in regulating and responding to altered DNA replication *in vivo*. When DNA synthesis is blocked, p53 inhibits entry into mitosis (29), and in several cell lines p53 is transcriptionally dysfunctional when DNA synthesis is blocked (30). p53 protein prevents mammalian DNA reduplication, following the release from the mitotic spindle checkpoint, in a transcription-independent manner (13). In addition, p53 inhibits the replication of Py and papillomavirus origin-containing DNAs in a cell-specific way: p53 represses replication of both viral origins in human and Chinese hamster cells, but fails to do so in mouse cells (31–33). Further evidence supporting the possibility for a direct interaction between p53 and the DNA replication machinery is provided by the observations that p53 is localized to foci of replication in the nuclei of herpes virus-infected cells (34) and that p53 is imported into the nucleus of *Xenopus* oocyte upon fertilization, although the first 12 rapid cell cycles occur without transcription of the genome (35).

To continue to explore possible alternative roles and functions of p53, in this study we tested the possibility that p53 affects the replication of damaged DNA templates. We found that p53 can block the initial stage of replication of a γ-irradiated plasmid containing the Py replication origin. Such inhibition requires both specific binding by the p53 core domain and non-specific binding by its C-terminus. We discuss the implications of our findings including the likelihood that the effects seen require DNA looping by p53.

MATERIALS AND METHODS

Plasmids

Vw16PyCAT contains 16 p53 binding sites (RGC; refers to the p53 binding site in the ribosomal gene cluster) on the late side of the Py replication origin and is the same plasmid used by Miller *et al.* and Vogelstein and Kinzler in their respective studies (28,36). pBE102 has been described previously and contains the A2 strain of Py wild-type sequences extending from the BclI site at nucleotide 5022 to the EcoRI site at nucleotide 1562 inserted into a pML vector containing neo coding sequences (37). To construct Vw16PyCATBSM1 (BSM: p53 binding site mutant) and Vw16PyCATBSM2, oligonucleotides 5′ CGCCTCCAAGGCGCCAGTTCTATT-TTGATAAATTGCCTCTGGAAGC 3′ and 5′ CGCCTCCA-AGGCGCCAGACATGTTTGGACAAGTTCCCTCTGGA-AGC 3′ were used as the forward primer, respectively, to mediate the PCR-directed mutagenesis. Oligonucleotide 5′ CCCTCGAGGTCGACGGTATCG 3′ (a pBluescript II SK sequence) was used as the reverse primer. Similarly, the same two forward primers and oligonucleotide 5′ ACAGGAG-ATCCTGCCCCGGCA 3′ (a neo sequence), as the reverse primer, were used to construct pBE102BSM1 and pBE102BSM2. PCR products of 588 and 814 bp were

recovered and digested with PstI/NarI or NarI, respectively. The 140 and 621 bp fragments were ligated with their respective vectors, PstI/NarI digestion product of Vw16Py-CAT and the 3.9 kb fragment recovered from NarI digestion of pBE102.

Proteins

Baculovirus-expressed HA-tagged p53 protein was purified as described (38). Active HA–p53 was immunopurified using PAb421 antibody, while latent HA–p53 and HA–p53ΔC30 were immunopurified using mAb12.CA5 antibody. Oxidized HA–p53 was prepared similarly except that extraction, elution and dialysis were done in buffers lacking DTT. Py large T antigen was immunopurified from baculovirus-infected Sf21 cells as described previously (26).

Replication of Py DNA *in vitro*

Extracts of mouse FM3A cells were prepared as described (26). Standard replication reaction mixtures (50 µl) contained 40 mM creatine phosphate (pH 8.0), 7 mM MgCl$_2$, 0.5 mM DTT, 4 mM ATP, 200 µM each CTP, UTP and GTP, 100 µM each TTP, dGTP and dCTP, 20 µM dATP, 0.03 µM [α-^{32}P]dATP and 100 µg of creatine kinase/ml. 300 ng template plasmid, 0.5–1 µg Py T antigen, FM3A cell extract (300–400 µg of protein) and reaction mixtures were incubated at 33°C for 3 h. Replication products were analyzed by either acid precipitation or phosphoimaging analysis of agarose gels, following DpnI treatment of purified replication products, with a Molecular Dynamics PhosphoImager. For replication of γ-irradiated pBE102, purified pBE102 plasmid was first γ-irradiated in water at a final concentration of 300 ng/µl and then used as template in replication reactions.

Electrophoretic mobility shift assay

EMSA was performed as described (39). The oligonucleotide probe containing 3-fold RGC site is as follows: 5′ AATTCCCCGGATCGCCTTGCCTGGACTTGCCTGGCC-TTGCCTTTTCGATCCGGGG 3′. The oligonucleotide containing wild-type or mutant p53 binding site was created by digesting the 621 bp NarI digestion product of pBE102, pBE102BSM1 or pBE102BSM2 with HinfI, and the resulting 411 bp oligonucleotide fragment, which represents Py sequence from nucleotide 5076 to 89, was gel-purified and end-labeled. Reaction mixtures (20 µl) contained 4 µl of 5× EMSA buffer [100 mM HEPES (pH 7.9), 125 mM KCl, 0.5 mM EDTA, 50% glycerol, 10 mM MgCl$_2$], 1 µl of 40 mM spermidine, 1 µl of 10 mM DTT, 1 µl of 0.5% NP-40, 1 µl of 60 mg/ml double-stranded poly(dI–dC), 2 µl of 1 mg/ml bovine serum albumin, 1 ng of ^{32}P-labeled probe DNA and proteins as indicated. Reaction mixtures were incubated for 20 min on ice and then subjected to electrophoresis on 4% native polyacrylamide gels at 4°C. Gels were dried and autoradiographed.

RESULTS

p53 requires its sequence-specific DNA binding function to inhibit the *in vitro* replication of polyoma origin DNA

We reported previously (28) that wild-type human p53 strongly inhibits the replication of a DNA plasmid

(Vw16PyCAT) containing multiple copies of a p53 binding site (RGC) cloned adjacent to the late side of the Py origin, while the replication of a DNA plasmid containing mutated binding sites, is not affected by p53 (please refer to Fig. 8 for depiction of Py ori and early and late sides). Moreover, in that study two tumor-derived core domain p53 mutants, Trp-248 and His-273, were shown to be unable to inhibit the replication of Vw16PyCAT. These findings implicated sequence-specific DNA binding by p53 as a requirement for its ability to repress Py ori-DNA replication. To confirm and extend these studies we examined the ability of various forms of p53, each differing in their ability to bind to DNA, to inhibit the replication of Vw16PyCAT in mouse FM3A cell extracts. Note that although FM3A cells have been reported to contain wild-type p53 whose protein levels are increased after UV irradiation (40), the extracts used in our experiments are from unstressed cells and contain little or no detectable p53 (data not shown).

It was shown previously by Jayaraman *et al.* (39) that N-terminally HA-tagged p53 protein that had been immuno-purified using an affinity column containing the C-terminal-specific monoclonal antibody PAb421, HA–p53 [PAb421], is more active in sequence-specific DNA binding than a similarly purified p53 protein preparation (HA–p53 [flu]) in which the affinity column contained the HA antibody (mAb12.CA5). The basis for this difference is not fully understood but may be related to the observations that interactions of PAb421 with the p53 C-terminus can in some cases stimulate its ability to bind to DNA (41). It has also been shown that p53 purified in the absence of reducing agents (HA–p53 [flu, –DTT]) is inert in DNA binding (39,42,43). In contrast, as is well-documented, p53 lacking the C-terminus (HA–p53ΔC30 [flu]) can bind extremely well to DNA (41,44). These four different forms of HA-tagged p53 were prepared and tested for their ability to affect replication of Vw16PyCAT Py ori-DNA. As expected, equivalent quantities of these p53 protein preparations, HA–p53ΔC30 [flu], HA–p53 [PAb421], HA–p53 [flu] and HA–p53 [flu, –DTT], have descending affinities for an oligonucleotide probe, which contains three copies of the RGC site (Fig. 1A and B). The abilities of the different forms of p53 protein to inhibit the replication of Vw16PyCAT *in vitro* as measured both by incorporation of labeled deoxynucleotide into progeny DNA (Fig. 1C) and DpnI-resistant DNA detected on a 1% vertical agarose gel (Fig. 1D) reflected their relative abilities to bind to DNA. Note that the presence of DpnI-resistant DNA suggested that all the dAMP incorporation measured was produced by replication synthesis. We then tested a core domain 'hot-spot' p53 mutant, HA–p53[His–175] [flu], as well as an N-terminally mutated form of p53, HA–p53[Gln–22/Ser–23] [flu], which has been shown to be defective in transactivation of p53 target genes but active in sequence-specific DNA binding (45). As shown in Figure 2, both wild-type HA–p53 [flu] and HA–p53[Gln–22/Ser–23] [flu] bound to the oligonucleotide probe containing three copies of RGC sites to similar extents, while, as expected, HA–p53[His–175] [flu] failed to interact with the RGC sites. Correspondingly, wild-type HA–p53 [flu] and HA–p53[Gln–22/Ser–23] [flu] inhibited replication of Vw16PyCAT in a dose-dependent fashion while HA–p53[His–175] [flu] failed to do so. Our results thus strongly support the conclusion that the essential feature required for p53 to block DNA replication

is specific binding to the Py ori-DNA template. Furthermore, the fact that HA–p53[Gln–22/Ser–23] [flu] functions like wild-type HA–p53 [flu] in inhibiting the replication of Vw16PyCAT suggests that this inhibition does not require interaction of the N-terminus with cellular proteins that bind to its activation domain.

A p53 binding site in the Py origin region is required for the inhibition of the replication of Vw16PyCAT by p53

During the course of our earlier study a consensus p53 binding site on the early side of the Py core origin, spanning nucleotides 66–85, was identified and we showed that p53 can protect this site from DNase I digestion (28). We did not, however, determine whether the interaction of p53 with this site was required for p53 to block replication of constructs containing binding sites on the late side of the origin. To determine the role of this endogenous p53 binding site in the inhibition of the replication of Vw16PyCAT by p53, two mutant plasmids, Vw16PyCATBSM1 (BSM: p53 binding site mutant) and Vw16PyCATBSM2, were constructed that have mutated forms of this endogenous p53 binding site (Fig. 3A). In PyORI-BSM1 the two sets of essential C and G nucleotides from the p53 consensus sequence were mutated to T and A, while PyORI-BSM2 was altered to conform to a 'perfect' p53 consensus sequence. To test the affinity of p53 protein to the three different sequences, we used two different approaches. Figure 3B shows the result of a gel mobility shift assay in which HA–p53 [flu] bound end-labeled 411 bp oligonucleotide fragments excised from the respective Vw16PyCAT derivatives (Py nucleotide 5076–89). Figure 3C shows a gel mobility shift assay with the three RGC sites containing oligonucleotide (as in Fig. 1B) as the labeled probe and increasing amounts of plasmid Vw16PyCAT, Vw16PyCATBSM1 or Vw16PyCATBSM2 as competitor DNAs. As expected, the results of both methods show that HA–p53 [flu] protein has the highest affinity to PyORI-BSM2 and a minimal affinity to PyORI-BSM1.

We then used the three different plasmids as the template in the Py ori-DNA replication assay. Interestingly, while in the absence of p53, Vw16PyCATBSM2 had a replication efficiency close to that of Vw16PyCAT, Vw16PyCATBSM1 replicated 3–4-fold more efficiently than the other two plasmids (data not shown). Since the endogenous p53 binding site is located between two T antigen binding consensus pentanucleotides spanning nucleotides 62–66 and 95–99, it may be that the weaker T-A base pairs constructed in PyORI-BSM1 make it easier for T antigen to unwind the origin DNA. In the presence of HA–p53 [flu] protein the replication of Vw16PyCATBSM2 was inhibited even more efficiently than the replication of Vw16PyCAT, while the replication of Vw16PyCATBSM1 was not affected at all by HA–p53 [flu] (Fig. 3D). Our results thus show that the ability of p53 to inhibit the replication of Vw16PyCAT closely correlates to the affinity of p53 for the endogenous p53 binding site, and they further support the conclusion that p53 inhibits the replication of Vw16PyCAT in a DNA binding-dependent manner.

p53 inhibits the replication of radiation damaged, but not intact, pBE102

It was of interest to expand these observations to ask what might be considered a more physiologically relevant question,
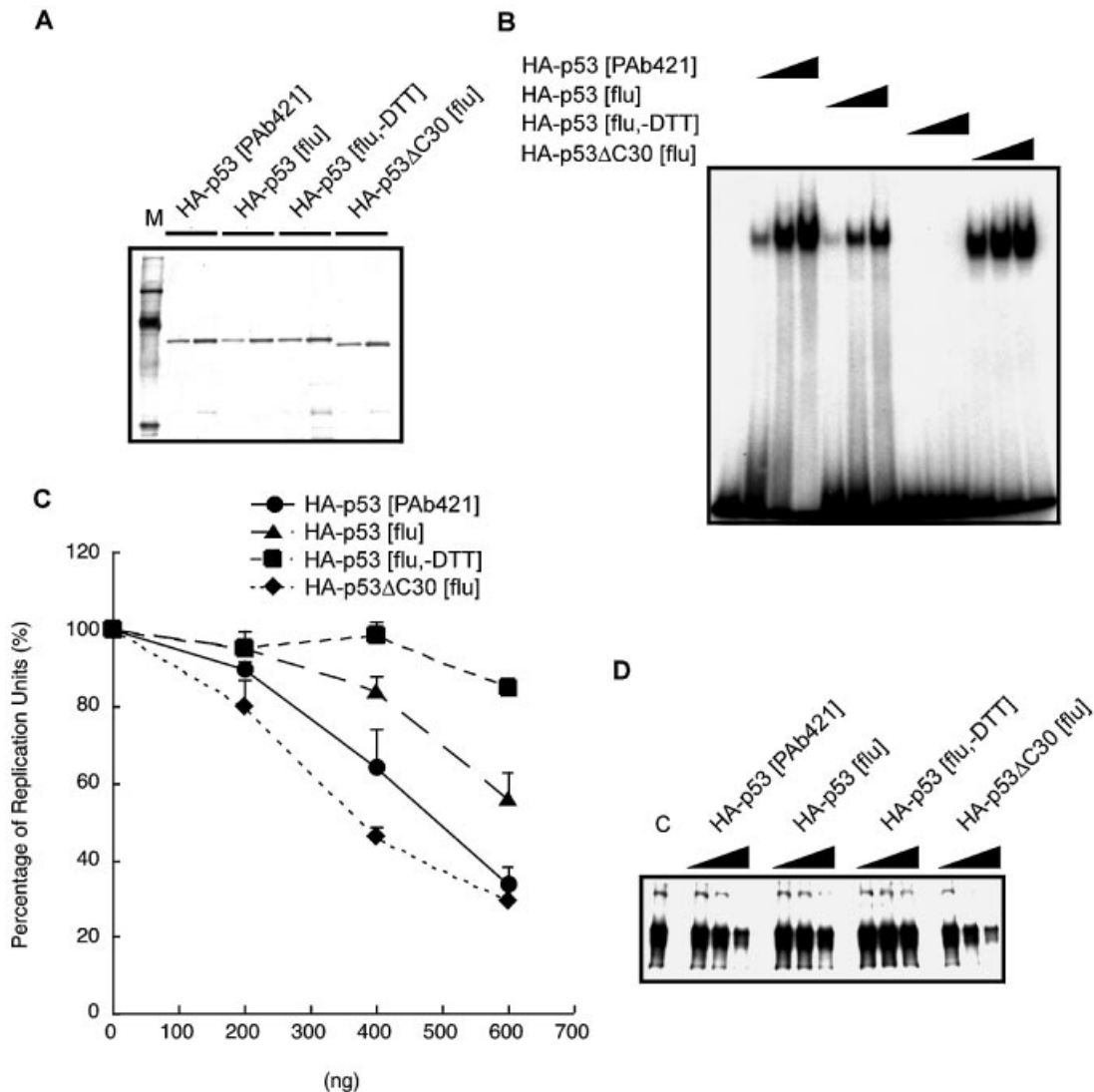
Figure 1. p53 inhibits replication of Py ori-DNA in a sequence-specific DNA binding-dependent manner. HA-tagged human p53 was purified from infected insect cells on either a PAb421 column HA–p53 [PAb421], or a mAb12.CA5 column HA–p53 [flu] with or without DTT. Similarly, flu-tagged p53ΔC30 (1–363) was purified on a mAb12.CA5 column. (**A**) 300 and 600 ng of each protein were run on a SDS–PAGE gel which was silver stained. M lane contains molecular mass marker proteins. (**B**) Increasing amounts of each protein (10, 20 or 40 ng) were added to EMSA reaction mixtures including as probe ^{32}P-end-labeled oligonucleotide containing three RGC p53 binding sites. (**C**) Increasing amounts (200, 400 or 600 ng) of each type of p53 protein were added to reaction mixtures containing 300 ng of Vw16PyCAT plasmid DNA and 600 ng of Py large T antigen. Replication was measured by acid precipitation of replicated DNA. The percentages represent the average of five separate experiments with bars showing the sample standard error. (**D**) Replication products from one experiment were purified and analyzed by DpnI digestion. C: control.

which is whether p53 can block the replication of damaged DNA. This is hypothetically feasible because p53 contains another autonomous DNA recognition region within its C-terminal domain that binds to several forms of DNA including DNA damaged by irradiation (for a review, see 10) as well as DNA with single or 4 nt gaps (J.Ahn, J.Zhou, and C.Prives, unpublished data). Thus, would DNA damage randomly introduced into the Py genome allow replication of Py DNA to be inhibited by p53? To this end, we γ-irradiated a plasmid pBE102, which contains the wild-type Py sequence extending from nucleotide 5022 to 1562 (37), and examined the ability of p53 to inhibit the replication of this plasmid. pBE102 contains the endogenous p53 binding site on the early side of the origin but lacks additional p53 binding sites and its

replication is not affected by p53 (26). As shown in Figure 4A and B, increasing doses of ionizing radiation reduced the ability of pBE102 to serve as a replication template. When HA–p53 [flu] was added to the reaction mixtures, replication of pBE102 that had been pre-treated with the same doses of γ-irradiation was further inhibited in a p53-concentration-dependent manner.

Although in the presence of T antigen, the majority of dAMP incorporation *in vitro* using γ-irradiated pBE102 as a template is likely due to replication synthesis, the possibility cannot be excluded that repair synthesis as detected by labeled deoxynucleotide incorporation could also be inhibited by p53. Therefore, DpnI resistance of the replication products was examined in order to distinguish replication synthesis from
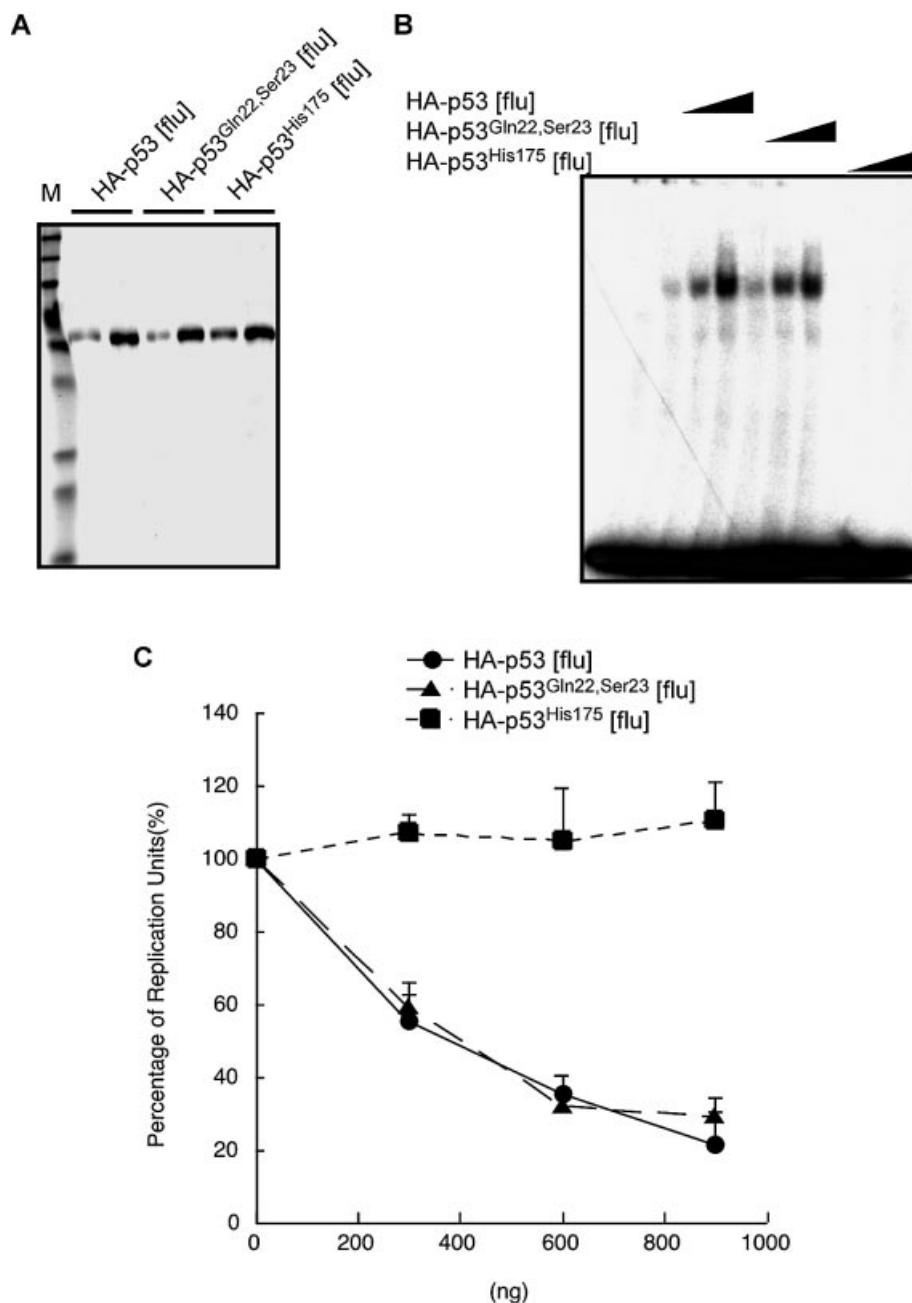
Figure 2. Wild-type and an N-terminal mutant p53Gln22/Ser23, but not the central core domain mutant p53His175, inhibit the replication of Py ori-DNA. (**A**) Baculovirus-expressed wild-type and mutant HA–p53 proteins (300 and 600 ng) were immunopurified on a mAb12.CA5 column and analyzed on a silver stained SDS–polyacrylamide gel as indicated. M lane contains molecular marker proteins. (**B**) Increasing amounts of each protein (10, 20 and 40 ng) were added to EMSA reaction mixtures containing end-labeled RGC probe as in Figure 1. (**C**) Effects of WT HA–p53 [flu], HA–p53Gln22/Ser23 [flu] and HA–p53His175 [flu] on the replication of plasmid Vw16PyCAT were compared. Replication was measured by acid precipitation of replicated DNA. The percentages represent the average of three separate experiments with bars showing the sample standard error.

repair synthesis. Interestingly, HA–p53 [flu] inhibited exclusively replication synthesis, while repair synthesis was not affected (Fig. 4C). To verify this result, we further examined the ability of HA–p53 [flu] to affect the repair of γ-irradiated pBE102 in the absence of T antigen where virtually all of the nucleotides were incorporated at sites of repair synthesis and here too HA–p53 [flu] did not affect these activities (Fig. 4D). Thus, our results show that p53 can specifically inhibit the replication but not the repair of a radiation damaged Py ori-DNA template.

Since the C-terminus is required for the interaction of p53 with irradiated DNA (46), we tested whether this region is necessary for the ability of p53 to block the replication of damaged ori-DNA. As shown in Figure 5A, among the four different forms of p53 protein used in Figure 1, only HA–p53 [PAb421] and HA–p53 [flu] inhibited the replication of γ-irradiated pBE102. In fact HA–p53 [flu] was relatively more effective in blocking the synthesis of damaged ori-DNA than of intact ori-DNA containing RGC binding sites. Neither HA–p53ΔC30 [flu], nor the oxidized HA–p53 [flu, –DTT],
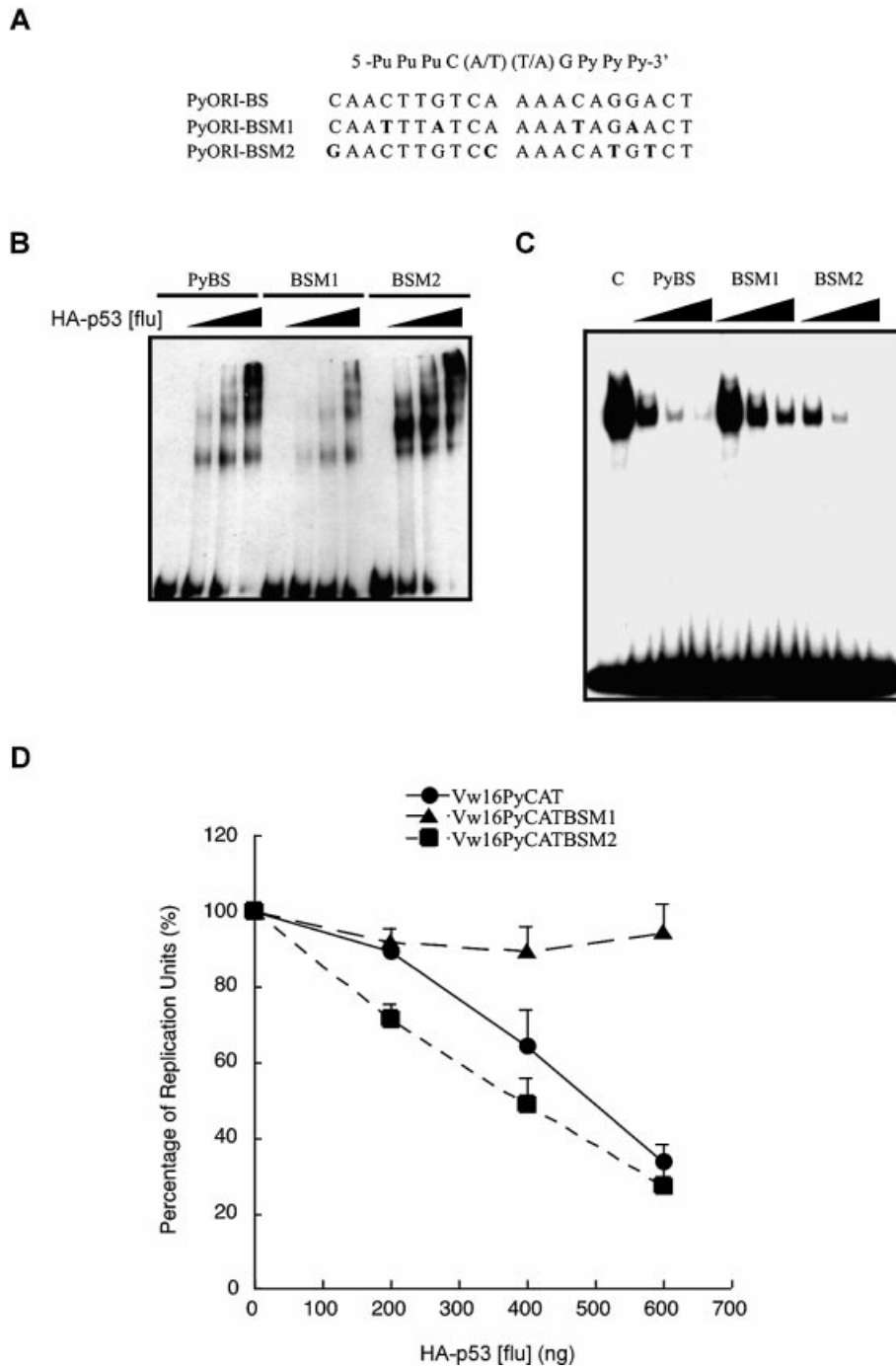
A

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                        5'-Pu Pu Pu C (A/T) (T/A) G Py Py Py-3'

PyORI-BS        CAACTTGTCA AAACAGGACT
PyORI-BSM1      CAATTTATCA AAATAGAACT
PyORI-BSM2      GAACTTGTCC AAACATGTCT
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B

C

D

Figure 3. The endogenous p53 binding site in the Py origin is required for p53 to inhibit the replication of Py ori-DNA. (**A**) Sequence of wild-type Py genome spanning Py nucleotides 66–85 (the endogenous p53 binding site, PyORI-BS) and two mutant forms of this sequence (PyORI-BSM1 and PyORI-BSM2) are shown below the p53 consensus binding sequence. Bold characters show the mutated deoxynucleotides. (**B**) Increasing amounts of purified HA–p53 [flu] (10, 20 and 40 ng) were added to EMSA reaction mixtures including end-labeled 411 bp oligonucleotide fragments spanning Py nucleotides 5076–89 containing either the wild-type Py sequence, PyORI-BSM1 or PyORI-BSM2 as indicated. (**C**) Increasing amounts of unlabeled plasmids Vw16PyCAT, Vw16PyCATBSM1 or Vw16PyCATBSM2 (100, 300 and 900 ng) were added to EMSA reaction mixtures containing 20 ng of purified HA–p53 [flu] protein and 1 ng of end-labeled RGC oligonucleotide probe. (**D**) Effects of HA–p53 [flu] on the replication of Vw16PyCAT, Vw16PyCATBSM1 and Vw16PyCATBSM2 were compared.

inhibited the replication of γ-irradiated pBE102. Thus, while the C-terminus of p53 is not required to block the replication of plasmids bearing p53 binding sites, it is indispensable for p53 inhibition of replication of damaged plasmids. The fact that HA–p53 [flu, –DTT] was inert, however, suggested that sequence-specific DNA binding by p53 is also a component of its repressive effect on replication of damaged ori-DNA. This conclusion is supported by the experiment described below.
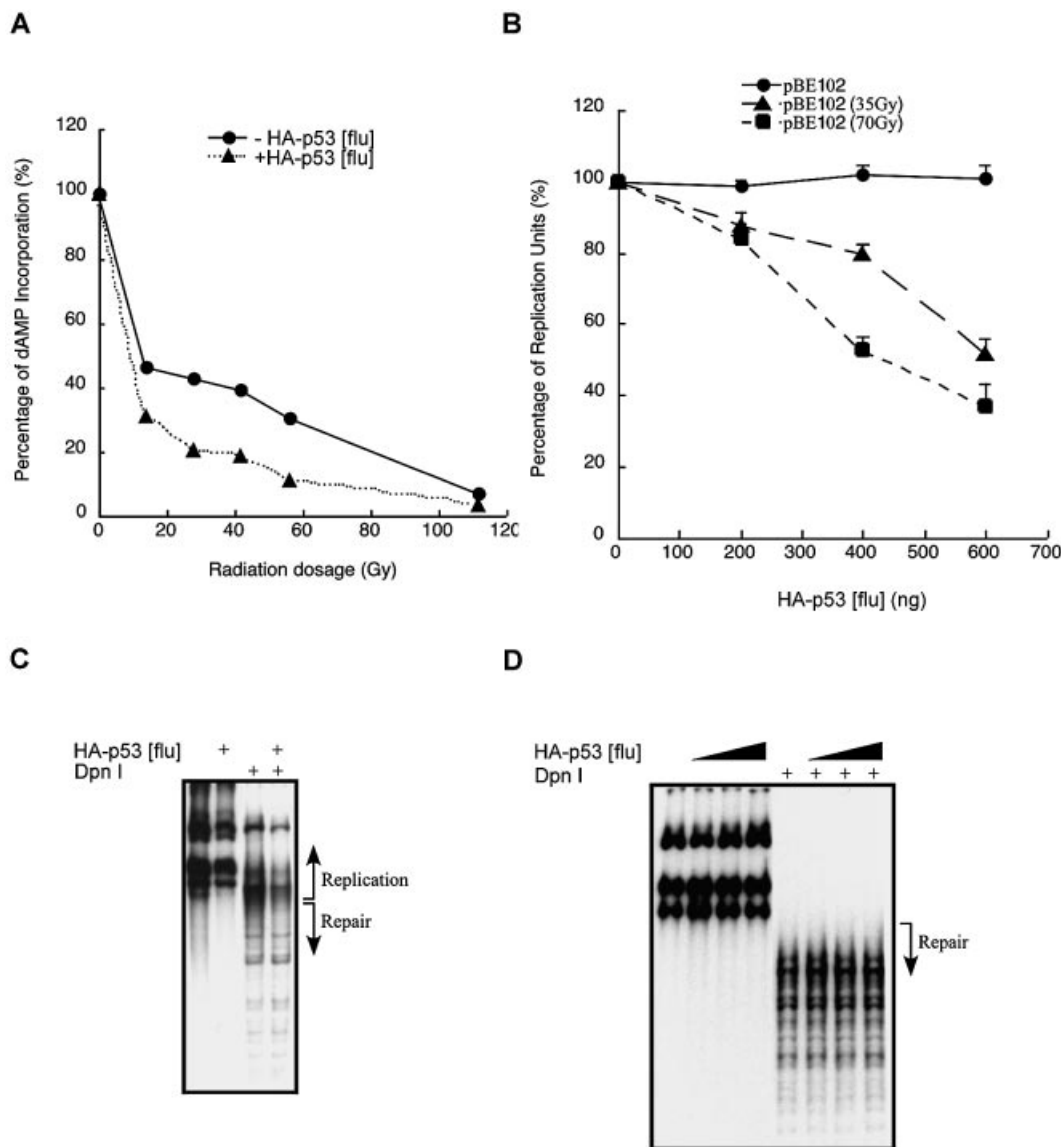
Figure 4. p53 inhibits replication but not repair synthesis of γ-irradiated Py-ori-DNA. (**A**) HA–p53 [flu] protein (600 ng) was added to DNA replication reaction mixtures in which plasmid pBE102 (300 ng) pre-treated with increasing dosages of γ-irradiation was used as the template. (**B**) Effects of HA–p53 [flu] protein on the replication of intact pBE102, pBE102 pre-treated with 35 Gy and 70 Gy γ-irradiation were compared; *n* = 5. (**C**) The replicated DNA from one experiment in which 600 ng of HA–p53 [flu] protein was added to the reaction mixture containing 300 ng of pBE102 pre-treated with 70 Gy γ-irradiation was purified and analyzed by DpnI digestion. The DpnI-resistant DNA molecules are replication products, while the low molecular weight, DpnI-digested DNA molecules are repair products. (**D**) Increasing amounts of HA–p53 [flu] protein (200, 400 and 600 ng) were added to replication reaction mixtures with 300 ng of γ-irradiated pBE102 (70 Gy) lacking Py T antigen. DNA products were purified and analyzed by DpnI digestion.

Inhibition of the replication of γ-irradiated pBE102 requires the endogenous p53 binding site

The fact that the endogenous p53 binding site on the late side of the origin is required for the inhibition of replication of plasmids bearing p53 binding sites, led to the question as to whether this site is also required for the inhibitory effect of p53 on γ-irradiated pBE102. To test this, plasmids pBE102BSM1 and pBE102BSM2 were constructed, which have the endogenous p53 binding site mutated to BSM1 and BSM2, respectively (as shown in Fig. 3A). Indeed, in contrast to wild-type or pBE102BSM2, the replication of γ-irradiated pBE102BSM1 was not inhibited by HA–p53 [flu] (Fig. 5C),

indicating that the endogenous p53 binding site is required for p53 to inhibit the replication of radiation damaged Py ori-DNA.

It remained possible that the ability of p53 to bind to irradiated DNA might facilitate its ability to inhibit replication of pBE102 by a mechanism that does not require irradiation of the pBE102 plasmid itself. Thus, p53 might bind simultaneously to an irradiated plasmid with its C-terminus and to another unirradiated plasmid through interaction of its central domain with the endogenous binding site and this would somehow prevent replication of the unirradiated plasmid. To test this we mixed untreated pBE102 and γ-irradiated pBluescript, which does not have the Py origin and therefore
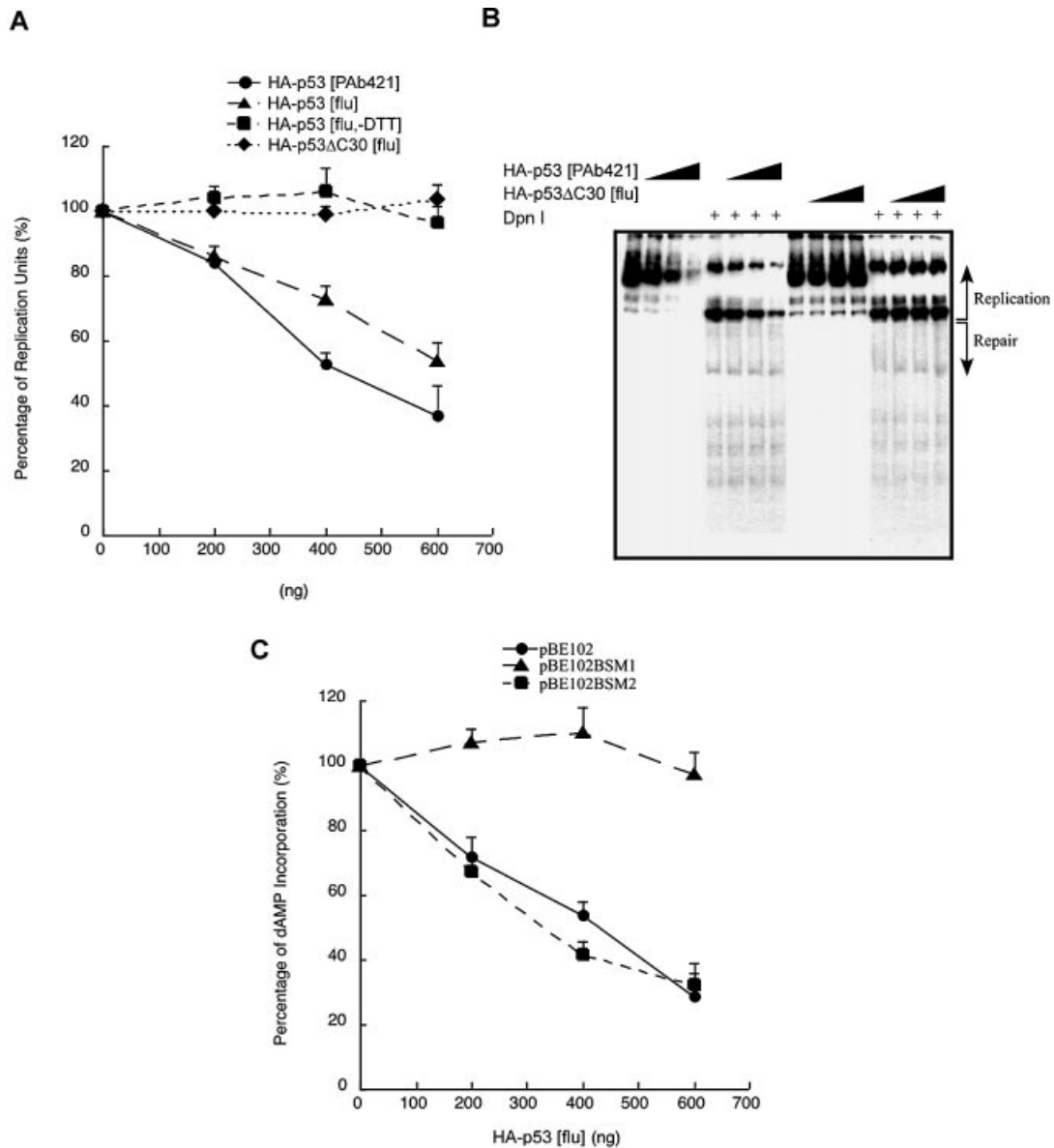
Figure 5. Inhibition of the replication of γ-irradiated Py ori-DNA requires the p53 C-terminus, as well as the endogenous p53 binding site in the Py origin. (**A**) Effects of the four different forms of p53 protein shown in Figure 1A on the replication of pBE102 (70 Gy) were compared; $n = 4$. (**B**) DNA products from one experiment using HA–p53 [PAb421] and HA–p53ΔC30 [flu] were analyzed by DpnI-digestion. The lower part of the gel containing the repair products was exposed three-times longer to autoradiography. (**C**) The effects of HA–p53 [flu] protein on the replication of pBE102, pBE102BSM1 and pBE102BSM2 were compared; $n = 4$.

does not support DNA replication with the Py large T antigen-complemented *in vitro* DNA replication system. It was observed that the endogenous p53 binding site and DNA lesion(s) caused by γ-irradiation do not work *in trans* in making DNA replication inhibited by HA–p53 [flu] (Fig. 6A). Similarly, HA–p53ΔC30 [flu] and the C-terminal domain of p53 (HA–p53[300–393] [flu]), which binds preferentially to the consensus site and DNA damage sites, respectively, do not work *in trans* either (Fig. 6B).

Inhibition of the replication of γ-irradiated pBE102 by p53 takes place in the initiation stage of DNA replication

It is feasible to design *in vitro* replication experiments to determine what step is being inhibited by p53. As was first demonstrated with SV40 ori-DNA replication, when reaction mixtures are deprived of deoxynucleotides and pre-incubated with T antigen, the pre-synthesis complex forms (47), but the complex is unable to begin elongation. It is thus possible to determine whether p53 inhibition of DNA replication occurs at the elongation or initiation stage by adding it to replication mixtures either before or after addition of deoxynucleotides. Pre-incubation with T antigen was carried out for 10 min and then HA–p53 [flu] protein (600 ng) was added and incubated for a further 10 min, followed by the addition of deoxynucleotides to allow elongation (Fig. 7). DNA replication after this treatment was compared with that in replication reaction mixtures when HA–p53 [flu] was added without pre-incubation (compare lane 3 with lane 5, lane 4 with lane 6).
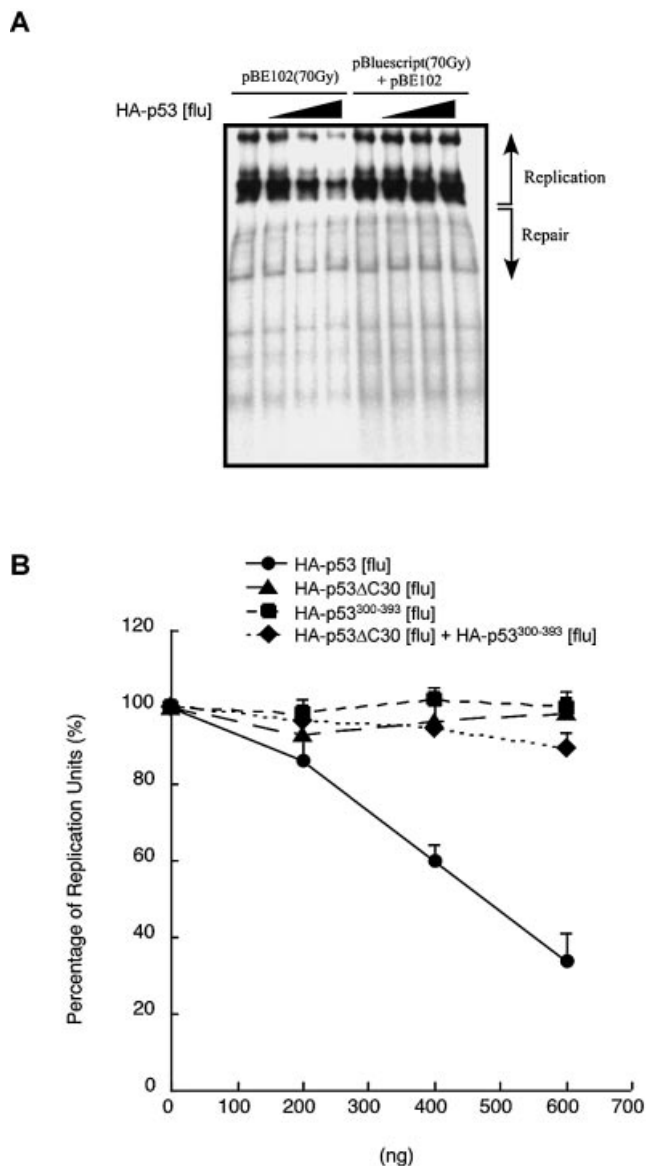
A

B

Figure 6. DNA lesions on the template are required *in cis* for the replication of pBE102 to be inhibited by p53 protein. (**A**) Increasing amounts of HA–p53 [flu] protein (200, 400 and 600 ng) were added to replication reaction mixtures containing either 300 ng of γ-irradiated pBE102 (70 Gy) or 200 ng of untreated pBE102 together with 200 ng of γ-irradiated pBluescript. DNA products were purified and analyzed by DpnI digestion. (**B**) Effects of the C-terminal domain of p53 (HA–p53[300–393] [flu]), HA–p53ΔC30 [flu] (200, 400 and 600 ng) or the combination of the two on the replication of γ-irradiated pBE102 (70 Gy) were compared with that of full-length HA–p53 [flu]; $n = 3$.

HA–p53 [flu] inhibited replication of both Vw16PyCAT and damaged pBE102 templates without T antigen pre-incubation. When T antigen was allowed to form the pre-synthesis complex at the origin; however, HA–p53 [flu] was no longer able to inhibit replication of either template (Fig. 7). The result that inhibition of replication of both types of template happens at the beginning of DNA replication suggests that p53 may inhibit replication of the two different types of templates through the same mechanism.
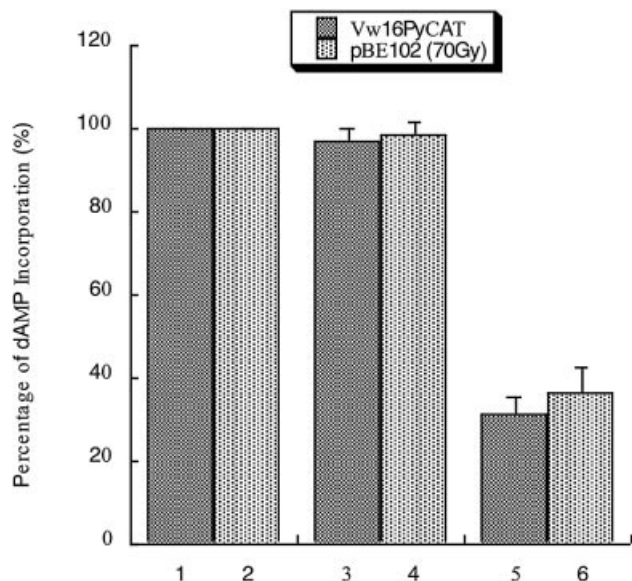

Figure 7. Inhibition of the replication of both Vw16PyCAT and γ-irradiated pBE102 takes place in the initiation stage of DNA replication. Replication reactions containing either Vw16PyCAT (lanes 1, 3 and 5) or γ-irradiated pBE102 (lanes 2, 4 and 6) were performed under two conditions: in lanes 3 and 4, 1 mg of Py T antigen was pre-incubated for 10 min with the template plasmid (300 ng) and FM3A cell extract (400 mg) at 33°C before HA–p53 [PAb421] (600 ng) and deoxynucleotides were added to the reaction mixtures; in lanes 5 and 6, the same amounts of T antigen and HA–p53 [PAb421] were added simultaneously into reaction mixtures. Lanes 1 and 4 are controls in which no p53 was added. Replication was determined as in previous figures; $n = 3$.

DISCUSSION

In this study we extended our previous conclusion that p53 must bind specifically to undamaged Py ori-DNA in order to block its replication and showed that the endogenous p53 binding site adjacent to the core Py origin is essential for this inhibition. Further, we found that DNA lesion(s) caused by ionizing radiation can also recruit p53 and thereby facilitate its inhibition of replication of the damaged DNA. Taken together our results show that for the replication of Py DNA to be inhibited by p53 protein, the endogenous p53 binding site on the early side of the origin, as well as other strong p53 interactions with the template, are required. These interactions can be mediated either by its central core or its C-terminal domains.

Do our results reflect the possibility that virtually any DNA binding protein when present in sufficiently high quantities may inhibit replication of radiation damaged ori-DNA? Arguing against this are our results showing that (28) p53 needs to be able to interact specifically with the endogenous p53 binding site on the early side of the ori as well as with DNA lesions caused by irradiation, (i) p53 needs both its specific and non-specific DNA binding domains to cause this inhibition, and (ii) p53 blocks specifically the initiation and not the elongation stage of ori-DNA synthesis. Thus, it is unlikely that p53 is blocking replication by random non-specific binding to the damaged DNA. It is also noteworthy that Ito *et al.* (48) showed that the transcription factor AP1 stimulates the initial stage of *in vitro* replication of a Py ori-DNA plasmid containing the AP1 binding site. Their result
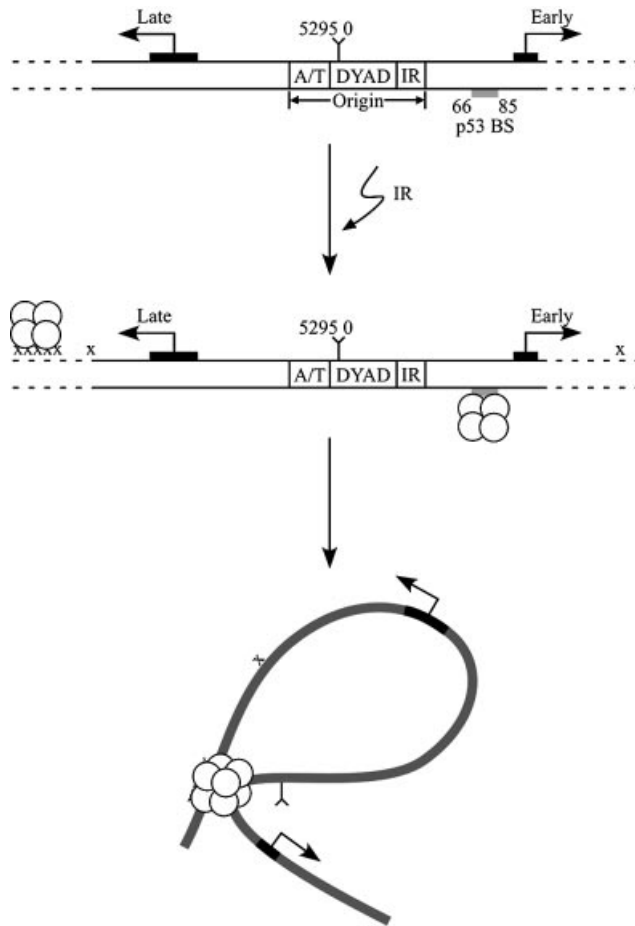
Figure 8. Model showing DNA looping mediated by p53. The Py core origin region (Origin), positioned between late and early regions, comprises an A/T-rich stretch (A/T), a region of dyad symmetry (DYAD) containing T antigen binding sites and an inverted repeat (IR). The endogenous p53 binding site (p53 BS) is located close to the origin (gray bar) on the early side. p53 protein is represented by the sphere tetramers. Exogenous p53 binding sites (either sequence-specific sites or sites of DNA damage) are represented by the 'x's.

argues against the possibility that a sequence-specific interactor with DNA in the vicinity of the Py ori will obligatorily inhibit its replication.

To explain the mechanism underlying the inhibition of Py DNA by p53, several possibilities can be suggested: (i) by binding to flanking consensus sites or lesion site(s), p53 may sequester away proteins which are essential for the replication machinery such as RP-A and T antigen; (ii) p53 binding to a site close to the replication origin, such as the endogenous p53 site of Py DNA, may block access of the replication initiation complex to the origin; (iii) p53 may also function as an anti-helicase (49) and counteract the helicase activity of T antigen by re-annealing denatured DNA around the origin; (iv) by binding to separate sites p53 may loop DNA around the origin and therefore produce steric hindrance to replication initiation. Our results presented in this paper strongly support the DNA looping model (Fig. 8), because both the endogenous p53 binding site on the early side and an *in cis* p53 binding site on the late side adjacent to the Py origin are required for the DNA replication to be inhibited by p53. It has been shown

previously that p53 binding to separate single consensus sequences can indeed bend and loop DNA, and DNA looping by p53 may play an important role in transactivation of certain promoters (50,51). So far, it remains to be tested whether p53 can also loop DNA by binding to separate DNA lesions, or as suggested by our result, by separately binding to a consensus sequence and a lesion site(s).

It was interesting to discover that when the endogenous p53 binding site adjacent to the Py origin was mutated to BSM1 (that cannot bind p53), the replication efficiency of the mutant plasmid was increased 3–4-fold (data not shown). The fact that the wild-type polyomavirus genome contains an endogenous p53 binding consensus site provides the intriguing possibility that polyomavirus may indeed use the host p53 protein to regulate amplicational replications. Amplicational replication of small DNA tumor viruses is normally initiated after entry of the viral genome into the cell nucleus, which leads to a rapid increase in copy number of the virus genome (32). Amplicational replication of the viral genome is essential for the establishment of viral infection but has to be controlled in order to avoid over-replication and unscheduled death of host cells prior to the synthesis of late genes and the production of infectious particles. It is tempting to speculate that polyomavirus uses p53 as a cellular factor to control amplicational replication. Further experiments will be required to test this possibility. Small DNA tumor viruses generally encode proteins that interact with and modulate the activity of p53 and thereby regulate p53-dependent apoptosis of the host cells. For example, the large T antigen of SV40 (26,52), E1B protein of adenovirus (53), x antigen of hepatitis B virus (54,55) and other viral proteins interact with p53 and thereby modulate p53-dependent apoptosis. So far no protein encoded by the Py genome has been identified that interacts with or regulates the activity of p53 protein. It supports the likelihood that polyomavirus uses p53 as a cellular factor to keep amplicational replication in check.

In the original conception of p53 as a checkpoint factor, it was proposed that DNA damage would induce a transient G_1 arrest in cells with wild-type but not mutant p53 to allow time for damaged DNA to be repaired before continuation of the cell cycle (56). It is tempting to speculate that p53 may participate directly in a pathway that stops or slows down DNA replication more rapidly than the transcription-dependent p21/E2F pathway. Although p53 is believed to interact with various forms of DNA damage through its C-terminus the biological function of this interaction has not been elucidated. In our study using ionizing radiation damaged Py ori-DNA plasmids we were able to demonstrate by *in vitro* experiments a potential new function for p53, namely that of direct inhibition of initiation of DNA replication in a DNA binding-dependent manner. It should be noted here that a p53-dependent DNA replication checkpoint has not been demonstrated (57), and in fact p53 has been shown to be transcriptionally impaired when it accumulates in blocked S-phase (30). What is not known, however, is whether at the very onset of S-phase radiation-damaged templates might be prevented from initiating replication as a result of direct interactions with p53. It is acknowledged that the replication of Py ori-DNA embodies several features that are distinct from the as yet poorly understood process of mammalian genomic DNA replication. Nevertheless, it is noteworthy that p53 could

only exert its effect on the initial stages of Py-ori DNA synthesis and, once initiated, p53 was ineffective in inhibiting ongoing replication. Thus, based both on our biochemical observations and the existing body of knowledge of p53 in cells, it is possible that p53 might serve to prevent the onset of S-phase both through its function as a transcription factor and its ability to bind to damaged DNA and prevent initiation of DNA synthesis. Once S-phase has initiated, it would follow that p53 would no longer be effective in inhibition of pre-initiated replication forks, even if stalled.

It is not clear how often sequence-specific p53 binding sites reside in the vicinity of mammalian replication origins. It is intriguing, however, that the two first cellular p53 binding sites to be identified were found in the vicinity of putative human replication origins (58). The ever-increasing number of transcriptional targets of p53 suggests that there may be large numbers of such binding sites in the genome. Besides polyomavirus, specific p53 binding sites have also been identified in the SV40 replication origin (59). It remains to be further studied whether DNA looping by p53 occurs and, as suggested by this study, plays functional roles regulating DNA replication in cells.

ACKNOWLEDGEMENTS

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