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Inhibition of Nucleotide Excision Repair by the Cyclin-dependent Kinase Inhibitor p21*

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Zhen-Qiang Pan‡§, Joyce T. Reardon§¶, Lei Li§‖, Hernan Flores-Rozas‡, Randy Legerski‖, Aziz Sancar¶, and Jerard Hurwitz‡**

From the ‡Graduate Program in Molecular Biology, Memorial Sloan-Kettering Cancer Center, New York, New York 10021, the ¶Department of Biochemistry and Biophysics, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599, and the ‖Department of Molecular Genetics, University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

p21, a p53-induced gene product that blocks cell cycle progression at the G_1 phase, interacts with both cyclin-dependent kinases and proliferating cell nuclear antigen (PCNA). PCNA functions as a processivity factor for DNA polymerases δ and ϵ and is required for both DNA replication and nucleotide excision repair. Previous studies have shown that p21 inhibits simian virus 40 (SV40) DNA replication in HeLa cell extracts by interacting with PCNA. In this report we show that p21 blocks nucleotide excision repair of DNA that has been damaged by either ultraviolet radiation or alkylating agents, and that this inhibition can be reversed following addition of PCNA. We have determined that p21 is more effective in blocking DNA resynthesis than in inhibiting the excision step.

We further show that a peptide derived from the carboxyl terminus of p21, which specifically interacts with PCNA, inhibits polymerase δ-catalyzed elongation of DNA chains almost stoichiometrically relative to the concentration of PCNA. When added at higher levels, this peptide also blocks both SV40 DNA replication and nucleotide excision repair in HeLa cell extracts. These results indicate that p21 interferes with the function of PCNA in both *in vitro* DNA replication and nucleotide excision repair.

In response to DNA damage, higher eukaryotic cells elevate the level of the tumor suppressor protein p53 (Maltzman and Czyzyk, 1984; Kastan *et al.*, 1991; Fritsche *et al.*, 1993; Hall *et al.*, 1993). This protein functions as a sequence-specific transcription factor that activates the synthesis of a number of proteins that act as cell cycle regulators. One such protein, p21 (also known as Cip1, WAF1, and Sdi1), binds to cyclin-dependent kinases (CDKs)[1] and inhibits their activity resulting in cell cycle arrest at the G_1 phase, presumably to allow for the repair of damaged DNA (El-Deiry *et al.*, 1993; Harper *et al.*, 1993; Gu *et al.*, 1993; Xiong *et al.*, 1993; Noda *et al.*, 1994). However, p53 is not the only transcriptional inducer of p21. It has recently been shown by several laboratories that p21 expression can be regulated independently of p53 during several situations including normal tissue development and cellular differentiation (Halevy *et al.*, 1995; Parker *et al.*, 1995; Macleod *et al.*, 1995). Thus, p21 appears to function as a general growth inhibitor in response to DNA damage and cellular differentiation.

Subsequent studies have shown that, in addition to its ability to bind to CDKs, p21 also directly interacts with proliferating cell nuclear antigen (PCNA) (Flores-Rozas *et al.*, 1994; Waga *et al.*, 1994). Chen *et al.* (1995) and Luo *et al.* (1995) have shown that these two distinct inhibitory activities of p21 reside in different domains of the protein. The NH_2-terminal domain of p21 contains the CDK inhibitory activity, while the COOH-terminal domain contains the PCNA binding and the DNA synthesis inhibitory activities. Luo *et al.* (1995) have shown that when separately expressed in R-1B/L17 cells, each of these domains is able to block DNA synthesis. This finding suggests that p21 may function to block entry into S phase by two different mechanisms: inactivating CDKs or neutralizing the function of PCNA.

PCNA acts as a processivity factor for both DNA polymerases δ and ϵ (pol δ and ϵ), and is required for DNA replication (for reviews, see Chalberg and Kelly (1989), Stillman (1989), and Hurwitz *et al.* (1990)). In the presence of a primer-binding protein, replication factor C (RF-C; also known as activator 1, A1; for reviews see Tsurimoto *et al.* (1990) and Hurwitz *et al.* (1990)), PCNA is loaded onto the DNA primer junction allowing the binding of pol δ or ϵ, which can then catalyze the elongation of DNA chains (Lee and Hurwitz, 1990; Burgers, 1991). These three proteins (PCNA, RF-C, and pol δ) constitute the pol δ holoenzyme, which is essential for the replication of simian virus 40 (SV40) DNA *in vitro*. p21 has been shown to inhibit both *in vitro* SV40 DNA replication and the elongation of primed DNA templates catalyzed by the pol δ holoenzyme (Flores-Rozas *et al.*, 1994; Waga *et al.*, 1994). In each system, the inhibition by p21 was found to be reversed by the addition of excess PCNA. Quantitative analysis of the binding of p21 to PCNA indicates that 1 mol of p21 binds to each monomer of PCNA. The active form of PCNA in DNA replication has been shown to be a trimer (Krishna *et al.*, 1994). The inhibition of the processive action of the pol δ holoenzyme by p21 has been shown to depend on both the molar ratio between p21 and PCNA and on the length of DNA replicated. Higher ratios of p21 to PCNA were found to be more inhibitory and the replication of longer chains was more sensitive to p21 than the extension of DNA chains over short lengths (Flores-Rozas *et al.*, 1994).

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§ These authors contributed equally to this work.

** Professor of the American Cancer Society. To whom correspondence should be addressed: Graduate Program in Molecular Biology, Memorial Sloan-Kettering Cancer Center, 1275 York Ave., Box 97, New York, NY 10021.

[1] The abbreviations used are: CDK, cyclin-dependent kinase; AAAF, *N*-acetoxy-2-acetylaminofluorine; PCNA, proliferating cell nuclear antigen; HSSB, human single-stranded DNA-binding protein; SV40, simian virus 40; T antigen, simian virus 40 large tumor antigen; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; pol, polymerase; CFE, cell-free extracts; RF-C, replication factor C.

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In addition to its role in replication, PCNA is also essential for DNA nucleotide excision repair (Shivji *et al.*, 1992; Nichols and Sancar, 1992). Nucleotide excision occurs through the introduction of nicks 3–5 nucleotides 3′ and 22–24 nucleotides 5′ to the site of damage, resulting in the removal of fragments 25–29 nucleotides in length (Huang *et al.*, 1992; Svoboda *et al.*, 1993). This gap is then filled in by pol δ or ϵ with the undamaged strand acting as the template (Zeng *et al.*, 1994; Aboussekhra *et al.*, 1995). PCNA is thought to act in conjunction with the DNA polymerase during nucleotide excision repair, undertaking a role similar to that observed in replication.

We have investigated the effect of p21 on PCNA-dependent DNA nucleotide excision repair *in vitro*. In this report, we present evidence that both the p21 protein and a peptide derived from the carboxyl end of p21 that binds PCNA specifically inhibit the repair of DNA that has been damaged by either alkylating agents or by ultraviolet (UV) radiation treatment. We further determined that this inhibition results from the ability of p21 to block the DNA resynthesis reaction following the excision of the damaged nucleotides. These findings are contradictory to previous reports by Li *et al.* (1994) and Shivji *et al.* (1994), who found that high levels of p21, which blocked DNA replication, did not affect nucleotide excision repair.

MATERIALS AND METHODS

Cell-free Extracts, Proteins, and Peptides—HeLa cell cytosolic and WI-L2 whole cell extracts, used in Figs. 1–6, were prepared as described previously by Wobbe *et al.* (1985) and Manley *et al.* (1980), respectively. HeLa S3 cells were obtained from the stock of Lineberger Comprehensive Cancer Center (University of North Carolina) and cell-free extracts (CFE) used in Figs. 7–9, were prepared by the method of Manley (Manley *et al.*, 1980). CFE were stored at 15 mg/ml in 25 mM Hepes-KOH, pH 7.9, 0.1 M KCl, 12 mM $MgCl_2$, 0.5 mM EDTA, 2 mM DTT, and 16% glycerol (v/v); CFE stored in this manner were active for at least three cycles of thawing and freezing at −80 °C.

PCNA, RF-C, HSSB, and pol δ were prepared as described previously (Kenny *et al.*, 1990; Lee *et al.*, 1991a). In all experiments, where indicated, the concentration of PCNA refers to the monomer form. The p21 peptide (GRKRRQTSMTDFYHSKRRLIFS) derived from the COOH terminus of p21 spans amino acids 139–160 and was synthesized in the Microchemistry Laboratory of Sloan-Kettering Cancer Institute and stored either as the solid or dissolved in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.1 M NaCl.

Escherichia coli UC6444 was transformed with plasmid pHIT5 in order to express the Nth protein (both were kindly provided by R. Cunningham, State University of New York, Albany), which was then isolated based on a procedure provided by R. Cunningham (Cunningham and Weiss, 1985).

Preparation of p21—The human histidine-tagged p21 (the construct was kindly provided by Dr. J. Massague, Sloan-Kettering Cancer Institute) was overproduced under the control of T7 RNA polymerase in *E. coli* (BL21-DE3). Freshly transformed bacteria were grown overnight in Luria broth containing 0.4% glucose and 0.5 mg/ml ampicillin at 37 °C. The overnight culture was diluted 1:100 with the same media (1 liter) and grown at 37 °C for 2.5 h to an A_{600} of 0.5. Isopropyl-1-thio-β-D-galactopyranoside (1 mM) was added, and the mixture was incubated for an additional 3.5 h. The cells were pelleted and lysed in 40 ml of buffer A (50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 5 mM imidazole, 1 mg/ml lysozyme, 2 mM PMSF, 4 μg/ml antipain, and 2 μg/ml leupeptin) for 30 min at 0 °C. After sonication (six repetitive 20-s treatments) and centrifugation at 39,000 × *g* for 20 min, the pellet containing more than 90% of the expressed p21, was washed once with 20 ml of buffer A and then resuspended in 20 ml of buffer B (50 mM Tris-HCl, pH 8.0, 20 mM DTT, 6 M urea, and 1 mM PMSF). After a brief sonication (three repetitive 15-s treatments) and centrifugation at 20,000 × *g* for 15 min, the supernatant (76 mg of protein, 19 ml) was used for the purification of p21. The 6 M urea extract was diluted 3-fold with buffer C (50 mM Tris-HCl, pH 7.8, 5 mM EDTA, 10% glycerol, 0.1% Triton X-100, 1 mM PMSF, 0.4 μg/ml antipain, and 0.2 μg/ml leupeptin), and chromatographed on a DEAE-Sepharose column (2.5 × 4 cm, 20 ml), equilibrated with 200 ml of buffer D (buffer C + 2 M urea + 5 mM DTT). The column was washed with 50 ml of buffer D. The flow-through and wash fractions were pooled (44 mg of protein, 110 ml) and dialyzed against 2 liters of buffer E (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.01% Nonidet P-40, 10% glycerol, 1 mM DTT, 0.1 mM PMSF, 0.2 μg/ml antipain, and 0.1 μg/ml leupeptin) containing 0.5 M NaCl for 12 h at 4 °C. The resulting fraction was completely soluble and was diluted 10-fold prior to chromatography through an S-Sepharose column (1.5 × 4 cm, 8 ml), equilibrated with 80 ml of buffer E plus 0.05 M NaCl. The protein was eluted with an 80-ml linear gradient of 0.05 to 1 M NaCl in the same buffer; 5.5 mg of p21 eluted from the column at 0.25 M NaCl. The peak fraction of p21 eluting from the S-Sepharose column (fraction 12, 0.96 mg of protein/ml; see text for details) was used in this study.

The p21 protein that bound to the DEAE-Sepharose column was eluted with buffer D containing 0.5 M NaCl, yielding 35 mg of protein. This fraction could be solubilized and further purified using the procedure described above.

Preparation of Repair Substrates—UV-damaged DNA substrate free of pyrimidine hydrates (used in Figs. 1–3 and 5) was prepared as described by Wood *et al.* (1988) by irradiating pUC18 plasmid DNA with UV light, followed by treatment with the *E. coli* Nth protein and purification through a 5–25% sucrose gradient. pVL1393 (9.6 kilobase pairs, Invitrogen) was used as a control plasmid.

The preparation of *N*-acetoxy-2-acetylaminofluorine (AAAF)-treated pBSIIKS plasmid DNA (used in experiments described in Fig. 6) was as described previously (Legerski *et al.*, 1977; Landegent *et al.*, 1984). pGEX2T was used as a control plasmid.

The DNA substrate used to assay both damage-specific DNA synthesis and excision (described in Figs. 7–9) was a double-stranded 140-mer containing a cholesterol "lesion" in place of a normal base at position 70 of one strand. The oligomers (Matsunaga *et al.*, 1995) used to prepare this substrate were purchased from Operon Technologies, Inc. or Midland Certified Reagent Co., T4 polynucleotide kinase and T4 DNA ligase were purchased from New England Biolabs, Inc., and [γ-^{32}P]ATP was from ICN. The substrate used to measure repair synthesis was prepared by mixing equimolar amounts of the two complementary 140-mers, one containing the cholesterol lesion, in a solution containing 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 2 mM $MgCl_2$; after heating at 90 °C for 5 min, the DNA was annealed by slow cooling to 25 °C over a period of several hours. The substrate was stored at −20 °C in annealing buffer and diluted prior to use in a buffer containing 1 mM Tris-HCl, pH 7.4, 1 mM NaCl, and 0.1 mM EDTA. The substrate used in the excision assay contains a ^{32}P-radiolabel 5′ to the lesion and was prepared as described previously (Matsunaga *et al.*, 1995). This substrate was stored and diluted as described above for the assay of repair synthesis.

Repair Assays—Repair assays with the UV-damaged DNA (described in Figs. 1–3 and 5) were carried out as described previously (Wood *et al.*, 1988; Sibghat-Ullah *et al.*, 1989). Reaction mixtures (40 μl) contained 40 mM creatine phosphate, pH 7.7, 50 μg/ml creatine kinase, 7 mM $MgCl_2$, 0.5 mM DTT, 2 mM ATP, 8 μM [α-^{32}P]dCTP (~29,000 cpm/pmol), 20 μM other dNTPs, 14.4 μg of bovine serum albumin, 70 mM KCl, 0.4 mM EDTA, 3.4% glycerol, 0.3 μg each of the UV-damaged and control plasmid DNA and HeLa cytosolic extracts as indicated. After incubation at 37 °C, as indicated, the mixture was treated with proteinase K (0.1 mg/ml) in the presence of 0.5% SDS, 5 mM EDTA, and 40 μg of glycogen at 37 °C for 30 min. DNA was recovered following phenol/chloroform extraction and ethanol precipitation and then linearized by *Bam*HI digestion at 37 °C for 60 min. The two plasmids present in the repair reactions were separated by 0.8% agarose gel electrophoresis and visualized by ethidium bromide staining. Following autoradiography, the radioactive DNA band was excised and quantitated by liquid scintillation counting.

Repair assays with AAAF-treated DNA (see Fig. 6) were carried out as described previously (Wood *et al.*, 1988). Reaction mixtures (50 μl) contained 192 μg of WI-L2 whole cell extract protein, 0.25 μg each of damaged and undamaged DNAs, and other components as described (Wood *et al.*, 1988). Histidine-tagged p21 (a gift of Dr. J. W. Harper; see Harper *et al.* (1993)) was added to the reaction mixture in the absence of DNA, and the mixture was incubated on ice for 30 min. After the addition of DNA, the reaction mixture was incubated at 30 °C for another 3 h.

In the repair assays described in Figs. 7–9, HeLa CFE (50 μg, 2 mg/ml) was mixed with substrate DNA (2 pmol) in reactions (25 μl) containing 35 mM Hepes (pH 7.9), 10 mM Tris-HCl (pH 7.5), 60 mM KCl, 40 mM NaCl, 5.6 mM $MgCl_2$, 0.4 mM EDTA, 0.8 mM DTT, 2 mM ATP, 3.2% glycerol, 0.2 mg/ml bovine serum albumin, 200 μM each of dCTP, dGTP, and dTTP, 8 μM dATP, and 4 μCi of [α-^{32}P]dATP (DuPont NEN) and incubated at 30 °C for 60 min. After the repair reaction, ^{32}P-radiolabeled 20-mer was added to each reaction as an internal control to monitor the recovery of substrate DNA. The mixture was deproteinized with proteinase K (0.2 mg/ml) followed by phenol, phenol:chloroform,

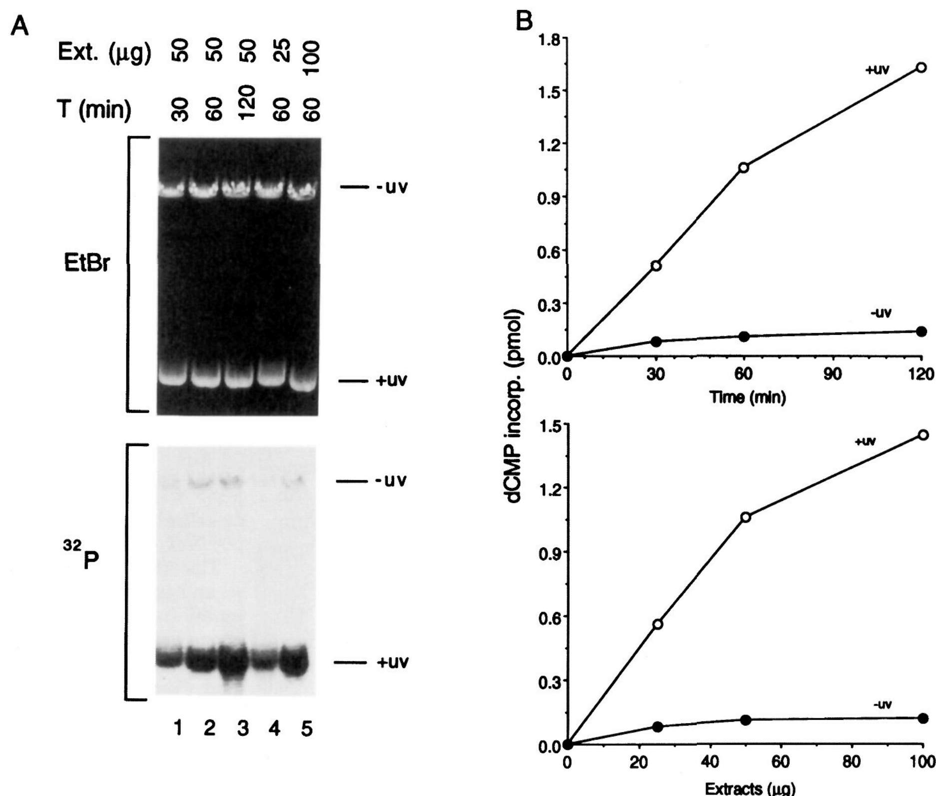

FIG. 1. **Influence of both time and the concentration of HeLa extracts on the repair of UV-damaged DNA.** *A*, reaction mixtures contained both UV-damaged and undamaged DNA and HeLa extracts as indicated. After incubation at 37 °C for times as indicated, the two plasmids were purified, linearized, and separated by agarose gel electrophoresis. The gel stained with ethidium bromide is shown at the *top*, and the autoradiogram is shown at the *bottom*. *B*, quantitation of [^{32}P]dCMP incorporated into each plasmid is shown. *Top*, 50 μg of extract protein was used. *Bottom*, reaction was incubated for 60 min.

and ether extractions, and the DNA was precipitated with ethanol in the presence of 40 μg of oyster glycogen. Recovered DNA was sequentially digested with *Pvu*II and *Hin*PI (New England Biolabs, Inc.), deproteinized, and precipitated. DNA was resuspended in formamide/dye mixture and resolved on a 12% denaturing polyacrylamide gel. Following autoradiography, the level of damage-specific repair synthesis was determined by scanning the autoradiographs using a Molecular Dynamics Computing Densitometer Series 300 instrument. Damage-specific repair synthesis was quantitated by determining the amount of labeled nucleotide incorporated into the 28-mer generated after *Pvu*II/*Hin*PI digestion. Nonspecific nicking and resynthesis of the undamaged strand was not observed (a labeled 30-mer would be generated as a product formed by the asymmetric *Pvu*II/*Hin*PI restriction pattern). The amount of repair synthesis was normalized for DNA recovery by quantitating the amount of 20-mer added as an internal control. When the influence of p21 protein or the p21 carboxyl-terminal peptide on repair synthesis was examined, reactions containing the inhibitors (diluted with a solution containing 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, and 1 mM EDTA) were preincubated for 15 min at 30 °C with HeLa CFE (50 μg). This was followed by the addition of the other components, and the DNA was processed as described for the basic repair synthesis reaction.

During experiments in which the inhibitory effect of p21 was reversed by PCNA, p21 was incubated with the indicated amounts of PCNA at 30 °C for 15 min and then HeLa CFE was added to a final concentration of 2 mg/ml. After a second 15-min incubation at 30 °C, substrate DNA, and other reaction components (as above, except that reactions contained 78 mM NaCl) were added to a final volume of 25 μl and, following a 60-min incubation at 30 °C, the DNA was processed as described for the basic repair synthesis reaction.

Excision Assay—The reaction conditions used were similar to those described for the repair synthesis assay, except that 3–5 fmol of substrate DNA (approximately 50,000 cpm) was used for each 25-μl reaction. pBR322 (150 ng) was included in each reaction, as well as 20 μM nucleotides followed by a 45-min incubation at 30 °C. Preincubation with p21 or the peptide was as described. Since the excision assay did not require restriction digestion, DNA recovered after the initial deproteinization and precipitation steps was resuspended in the formamide/dye mixture and resolved on a 12% denaturing polyacrylamide gel. Quantitation of excision (oligomers in the 23–30-nucleotide range) relative to the recovery of the added substrate was determined by either scanning autoradiographs with a Molecular Dynamics Computing Densitometer or by scanning dried gels with an AMBIS Systems Scanner.

DNA Replication Assays—SV40 DNA replication and the elongation of singly primed M13 DNA were carried out as described previously (Wobbe *et al.*, 1985; Flores-Rozas *et al.*, 1994).

RESULTS

Influence of p21 on Repair of UV-damaged DNA—UV damage to DNA leads to the formation of pyrimidine dimers and 6–4 photoproducts that are primarily repaired by the nucleotide excision pathway in mammalian cells (for reviews, see Sancar (1994) and Friedberg *et al.* (1995)). During nucleotide excision repair, the damage is removed by dual incisions in the form of 27–29-nucleotide-long oligomers (Huang *et al.*, 1992) and the resulting gap is filled in by DNA polymerases. PCNA has been shown to play an essential role in the repair of UV-damaged DNA (Shivji *et al.*, 1992; Nichols and Sancar, 1992). Repair can be quantified by measuring either the level of the excised fragment (excision assay; Huang *et al.*, 1992) or the level of incorporation of radiolabeled nucleotides (repair synthesis assay; Wood *et al.*, 1988; Sibghat-Ullah *et al.*, 1989). In order to assess the influence of p21 on the PCNA-dependent repair reaction, we initially examined the efficiency of the repair synthesis reaction as a function of both time and the concentration of HeLa extract added for the repair of UV-damaged DNA. As shown in Fig. 1, in the presence of 1.25 mg/ml HeLa extract, the rate of the repair of UV-damaged DNA was linear up to 60 min and continued to increase for at least 2 h.

The influence of p21 on the repair of UV-damaged DNA was examined (Fig. 2). For this purpose, the bacterial-expressed p21 protein was chromatographed through an S-Sepharose column and eluted using a salt gradient (see "Materials and Methods"). The elution profile of p21, measured by SDS-PAGE analysis (Fig. 2, *panel A*), and the inhibition of repair activity by aliquots of the eluted fractions (Fig. 2, *panel B*) were compared. These results were quantitated as shown in Fig. 2 (*panel C*). The peak of p21 eluted in fractions 12 and 14, coincident with the peak of inhibition of the repair reaction.

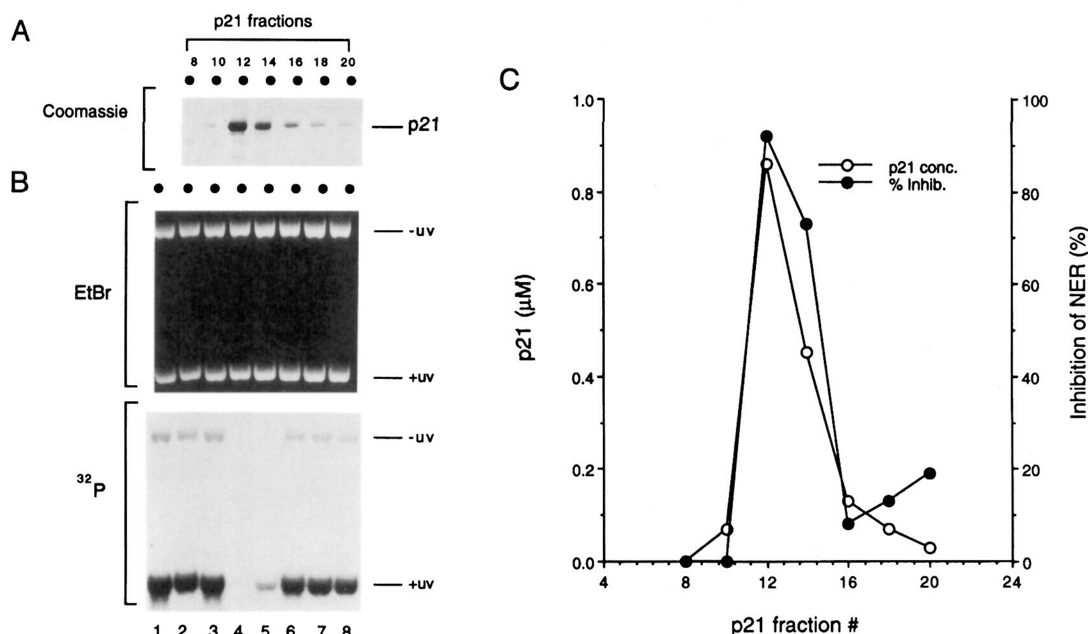

FIG. 2. **p21 inhibits repair of UV-damaged DNA.** *A*, SDS-PAGE analysis of the fractions isolated after S-Sepharose chromatography of p21. The p21 protein bound to the S-Sepharose column was eluted with a linear salt gradient as described under "Materials and Methods." The S-Sepharose eluted fractions (3 μl) were subjected to SDS-PAGE and stained with 0.1% Coomassie Brilliant Blue. The *numbers* at the *top* of the gel indicate the fractions analyzed. The position of the p21 protein is indicated. *B*, effect of p21 fractions eluted from the S-Sepharose column on the repair of UV-damaged DNA. Reactions contained none (*lane 1*) or 0.75 μl of the S-Sepharose fractions (*lanes 2–8*, as indicated), HeLa extract (1.26 mg of protein/ml), and other components as described under "Materials and Methods." After incubation at 37 °C for 30 min, reactions were stopped and analyzed as described under "Materials and Methods." Both the ethidium bromide staining and autoradiogram of the gel are shown. *C*, quantitation and comparison of the concentration of p21 in the various reactions and the inhibition of repair indicated as a percentage. In the absence of p21, 0.5 pmol of [^{32}P]dCMP was incorporated into the UV-damaged plasmid (representing 100% activity).

If the p21-mediated inhibition is due to its direct interaction with PCNA, addition of excess PCNA to the repair reaction should overcome this inhibition. An example of such an experiment is shown in Fig. 3*A*. In the presence of 0.42 μM p21, the repair reaction was inhibited 65% (Fig. 3*A*, *lane 2*), while at 0.84 μM p21, the reaction was reduced by 90% (*lane 6*). Addition of 0.17 μM PCNA (in its monomer form) to reactions containing 0.42 μM p21 almost completely reversed the inhibition (*lane 3*). In the presence of the higher level of p21 (0.84 μM), inhibition of repair was only partially overcome by addition of PCNA even at PCNA concentrations approaching 1 μM. The addition of PCNA in the absence of p21 did not significantly affect the repair reaction (Fig. 3*A*, *lanes 10–12*). These results indicate that p21 inhibits the repair of UV-damaged DNA and that this effect can be overcome following the addition of excess PCNA. However, the extent of the reversal depends on the p21 concentration used.

A PCNA-interacting Peptide of p21 Inhibits DNA Replication and the Repair of UV-damaged DNA—p21 has been shown to contain two distinct inhibitory domains. The NH_2-terminal region binds to and inhibits various CDKs, whereas the carboxyl domain binds to PCNA (Chen *et al.*, 1995; Luo *et al.*, 1995). These findings explain why p21 has been found complexed with CDK, cyclin, and PCNA (Xiong *et al.*, 1992). *In vitro* studies have shown that addition of CDK/cyclin A did not alter the inhibitory action of p21 in the PCNA-dependent, pol δ-catalyzed elongation of primed DNA templates. Experiments carried out with the cloned NH_2-terminal domain of p21 (codon 1–75), which inhibits CDKs, and the carboxyl-terminal domain of p21 (codons 76–164), which inhibits DNA synthesis, substantiate the conclusion that each domain can act independently (Luo *et al.*, 1995).

More recently, Warbrick *et al.* (1995) have shown that a peptide derived from the carboxyl domain of p21 interacts with PCNA and inhibits SV40 DNA replication catalyzed by crude cytosolic extracts. We have confirmed these observations using a peptide of 22 amino acid spanning codons 139–160 of p21. As shown, this highly basic peptide markedly inhibited the pol δ-holoenzyme system at concentrations even lower than those observed with p21 (Fig. 4*A*, see Table I). In the presence of 0.17 μM PCNA (monomer), 0.17 μM peptide markedly inhibited the elongation of singly primed M13 DNA template (*lane 5*). This inhibition was completely reversed by the addition of excess PCNA (0.85 μM, *lane 7*). In contrast to the near stoichiometric action of the peptide with the purified system, inhibition of the SV40 replication reaction with crude extracts required substantially more peptide and the extent of inhibition depended on the concentration of crude HeLa cytosolic extract added. In the presence of 2, 5, and 10 mg of extract protein/ml, 50% inhibition of SV40 replication was observed with 25, 47, and 86 μM peptide, respectively (Fig. 4*B*). These levels were substantially higher than the level of intact p21 required to inhibit SV40 replication in crude extracts (see Table I for a comparison). The reasons for this discrepancy are unclear. The possibility that the peptide was degraded by proteolysis in crude extracts was addressed. However, the addition of protease inhibitors did not decrease the amount of peptide needed to inhibit replication. Similar observations with crude extracts were made with the carboxyl terminus of p21 (codons 76–164).[2]

The 22-amino acid peptide derived from the carboxyl domain of p21 inhibited the repair of UV-damaged DNA by HeLa extracts in a manner analogous to that observed with the SV40 DNA replication system (Fig. 5*A*). In the presence of 0.63 and 1.26 mg of extract protein/ml, the repair reaction was inhibited 50% by 7.7 and 29 μM of the peptide, respectively, levels slightly higher than those required to inhibit the replication of SV40 DNA. Nonspecific peptides, C1 and C2 (described in the

[2] Y. Luo, J. Hurwitz, and J. Massague, unpublished results.

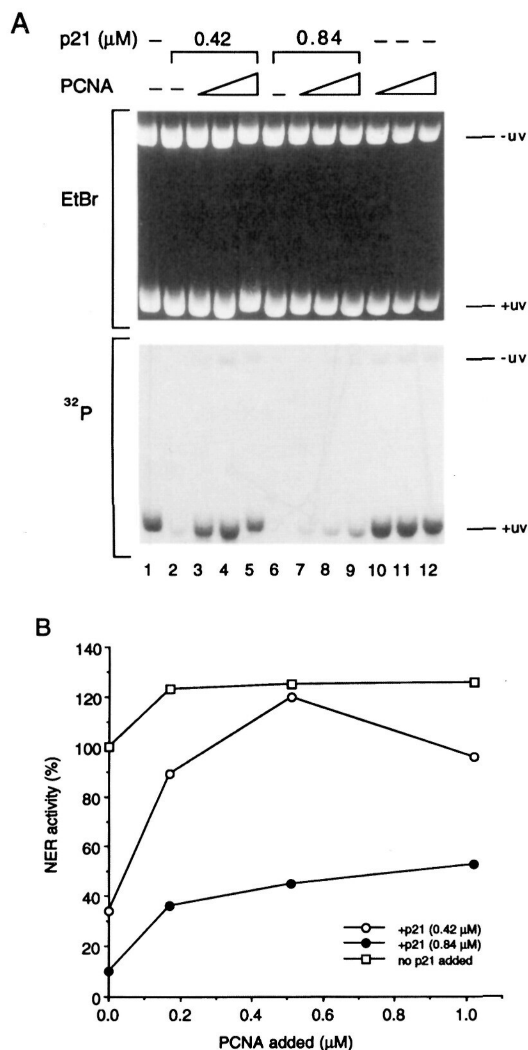

FIG. 3. **PCNA addition reversed the inhibitory effects of p21 on the repair of UV-damaged DNA.** *A*, all reactions contained HeLa extracts (1.25 mg of protein/ml) and other components as described under "Materials and Methods." The addition of p21 was as follows: none (*lanes 1* and *10–12*), 0.42 μM (*lanes 2–5*), or 0.84 μM (*lanes 6–9*). In experiments in which the effects of the addition of exogenous PCNA were examined, the amount of PCNA (as a monomer) added was 0.17 μM (*lanes 3, 7,* and *10*), 0.54 μM (*lanes 4, 8,* and *11*), or 1.08 μM (*lanes 5, 9,* and *12*). After incubation at 37 °C for 30 min, reactions were stopped and analyzed as described under "Materials and Methods." Both the ethidium bromide staining and autoradiogram of the gel are shown. *B*, quantitation of the experiment shown in *A*. In the absence of p21, 0.5 pmol of [^{32}P]dCMP was incorporated into the UV-damaged plasmid (representing 100% activity).

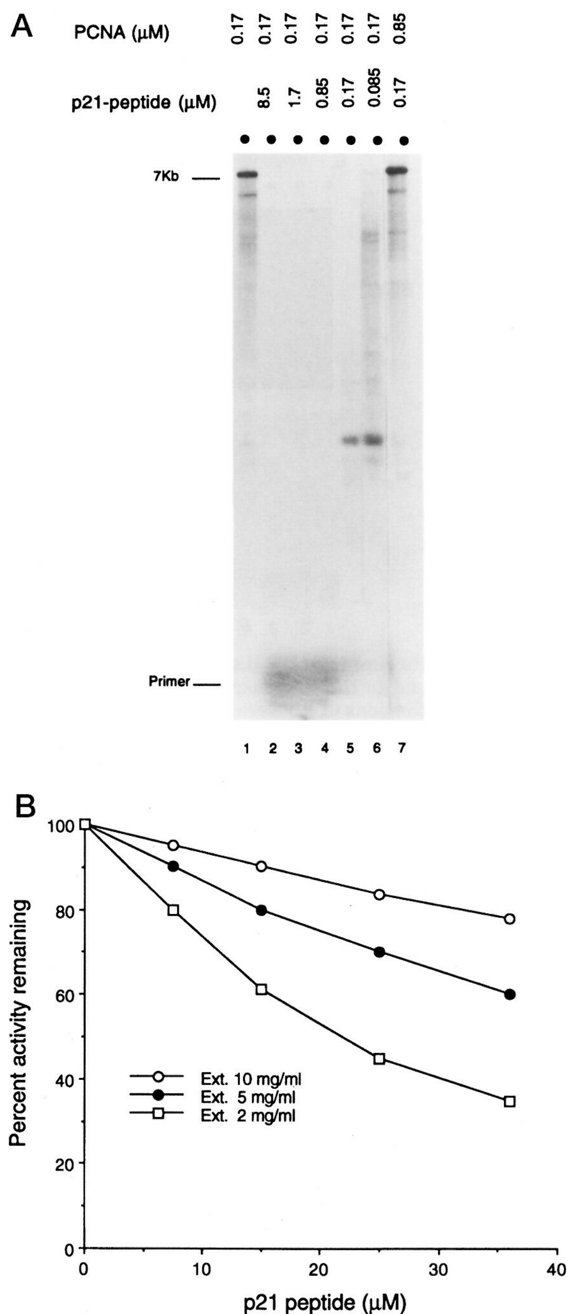

FIG. 4. **The influence of the p21 carboxyl-terminal peptide on the elongation of primed DNA templates and SV40 DNA replication.** *A*, the p21 carboxyl-terminal peptide inhibits elongation of labeled singly primed DNA by the pol δ holoenzyme. Reaction mixtures (10 μl) contained 30 mM Tris-HCl, pH 7.8, 2 mM DTT, 1.5 μg of bovine serum albumin, 33.3 μM dNTPs, 2 mM ATP, 7 mM $MgCl_2$, 5.1 fmol of singly primed M13 DNA (2600 cpm/fmol), 0.25 μg of HSSB, 0.075 unit of RF-C, 0.05 unit of pol δ, and PCNA and p21 carboxyl-terminal peptide in amounts indicated. Reaction mixtures lacking RF-C and pol δ were incubated for 5 min at 37 °C and cooled to 0 °C, and then RF-C and pol δ were added. After 20 min at 37 °C, reaction mixtures were adjusted to 10 mM EDTA, loading dye containing SDS was added, and the mixtures were subjected to alkaline agarose gel (1.5%) electrophoresis, dried, and autoradiographed. *B*, the p21 carboxyl-terminal peptide inhibits SV40 DNA replication by crude extracts of HeLa cells. Replication of SV40 DNA by crude extracts was carried out in reaction mixtures (10 μl) containing 75 ng of pSV01ΔEP, 0.32 μg of SV40 T antigen, 70 ng of HSSB, crude extract from HeLa cells and the p21 carboxyl-terminal peptide as indicated, and other reagents as described previously (Wobbe *et al.*, 1985). Reactions were incubated for 60 min at 37 °C, and the amount of acid-insoluble radioactivity formed (using [α-^{32}P]dCTP) was measured. In the absence of p21 peptide, 9.22, 7.74, and 3.29 pmol of dCMP were incorporated with 10, 5, and 2 mg of protein/ml, respectively.

legend to Fig. 5*A*), at comparable levels had no effect on the repair reaction. These observations indicate that the PCNA-interacting region of p21 alone is capable of inhibiting the repair reaction in the absence of the CDK-inhibiting domain, albeit at higher levels. At the high levels of peptide used, no attempts was made to reverse the inhibition with additional PCNA.

The Repair of DNA Damaged by Alkylating Agents Is Inhibited by p21—Since the repair of UV-damaged DNA is inhibited by p21, we determined whether this was true of DNA damaged by other agents. For this purpose, DNA alkylated by exposure to AAAF, which leads to guanine-substituted adducts was examined (Fig. 6*A*). In these experiments, WI-L2 whole cell extracts were used as the source of repair factors. As shown, at relatively high levels of p21 (between 0.38 and 0.76 μM) the repair of alkylated DNA was inhibited (quantitation of the

TABLE I

Summary of effects of p21 and the p21 carboxyl-terminal peptide on nucleotide excision repair and DNA replication

The data presented here summarize the effects of p21 and the p21 carboxyl-terminal peptide on both excision and resynthesis steps of nucleotide excision repair, SV40 DNA replication, and pol δ holoenzyme-catalyzed elongation of primed DNA templates.

	Nucleotide excision repair				SV40 replication	Pol δ holoenzyme-catalyzed DNA elongation
	Resynthesis			Excision		
Lesions in DNA	UV (6–4)[a]	AAAF[b]	Cholesterol	Cholesterol		
Extracts	HeLa	WI-L2	HeLa	HeLa	HeLa	
Purified enzymes						Pol δ, PCNA, RF-C, and HSSB
Protein (mg/ml)	1.26	3.84	2.00	2.00	4.65	PCNA (0.17 μM)
p21 for 50% inhibition (μM)	0.34	0.64	0.27	2	0.1–0.2	0.3
p21 peptide for 50% inhibition (μM)	29	ND[c]	25	No effect	47	0.06

[a] (6–4), (6–4) photoproducts. Most of the repair synthesis in UV-irradiated DNA is caused by (6–4) photoproducts, not pyrimidine dimers (Sibghat-Ullah and Sancar, 1990).

[b] AAAF, *N*-acetoxy-2-acetylaminofluorine.

[c] ND, not determined.

results is shown in Fig. 6*B*). The concentration of PCNA present in WI-L2 extracts was determined by quantitative Western blotting analysis using enhanced chemiluminescence and densitometric analysis. In comparison to known amounts of PCNA loaded on the same gel, it was determined that 1 μg of whole cell extract contained approximately 1.25 ng of PCNA (data not presented). Based on this analysis, each reaction described in Fig. 6 contained 0.17 μM PCNA (monomer). Thus, at a ratio of p21:PCNA of 0.4/0.17, the repair of AAAF-damaged DNA was inhibited by 20%; when the amount of p21 was increased 2-fold, the repair reaction was inhibited 78%.

The repair of AAAF-DNA can also be carried out by HeLa cytosolic extracts, and this reaction was inhibited by p21 in a manner analogous to that observed with WI-L2 whole cell extracts (data not shown). Excess PCNA reversed the inhibition (data not presented).

Influence of p21 on Excision and Damage-specific DNA Synthesis—Repair synthesis, assayed with randomly damaged DNA, often yields a "signal" due to DNA damage but not solely associated with repair (Biggerstaff and Wood, 1992; Reardon *et al.*, 1993). In order to avoid this problem, we tested the effects of p21 on excision and repair of DNA containing a single base lesion. Using a double-stranded 140-mer containing a cholesterol lesion as a substrate (a schematic drawing of the substrate is shown in Fig. 7*A*), we assayed both damage-specific DNA synthesis and excision of the damaged region. Damage-specific synthesis was assayed by measuring the incorporation of ^{32}P-labeled dAMP into a 28-mer (encompassing about 80% of the repair patch), derived from the repair of the damaged strand after restriction with *Pvu*II and *Hin*PI, within which the cholesterol lesion is located and removed by the excision nucleases. The excision assay, as described under "Materials and Methods," measured the release of an oligonucleotide (27-mer) from a 140-base pair duplex that contained a ^{32}P-labeled nucleotide specifically inserted within a region containing cholesterol in lieu of a base (Matsunaga *et al.*, 1995).

As shown in Fig. 7 (*B* and *C*), 0.27 μM of p21 inhibited damage-specific DNA synthesis by 50%, while approximately 2 μM of p21 was required to inhibit the excision step by 50%. The effect of the p21 carboxyl-terminal peptide on both resynthesis and excision steps was also determined (Fig. 8). Although the p21 peptide inhibited resynthesis 50% at 20 μM, no effect was observed in the excision assay at 40 μM, a concentration that almost totally inhibited the resynthesis step.

The inhibition of resynthesis by p21 was reversed by exogenous PCNA addition (Fig. 9). The slight stimulation of resynthesis observed at low concentrations of PCNA (Fig. 9*A*, *lane 4*) is most likely an experimental artifact. Reversal of the inhibition of the excision step was not attempted because of the high concentration of p21 required for 50% inhibition. It has been previously shown with partially purified fractions that PCNA is required for both the excision and resynthesis steps (Nichols and Sancar, 1992). The results presented here indicate that the resynthesis step is more sensitive to p21 than the excision step. In keeping with these observations, recent studies with highly purified fractions isolated from higher eukaryotes (Mu *et al.*, 1995) and from *Saccharomyces cerevisiae* (Guzder *et al.*, 1995) indicate that PCNA plays no role in the excision steps. It is interesting to note that with the highly purified excinuclease systems examined, the release of the excised oligonucleotide was effected by denaturing conditions. This raises the possibility that the recycling of the excision process may be stimulated by the PCNA-dependent repair synthesis observed with partially purified fractions, as previously suggested (Nichols and Sancar, 1992).

DISCUSSION

The results presented here (summarized in Table I) demonstrate that both p21 and a peptide derived from the carboxyl end of p21 that binds PCNA specifically inhibit the repair of DNA damaged by either alkylating agents or UV radiation treatment. We further determined that this inhibition is due to the blockage of the resynthesis reaction following the excision step. These observations, along with those reported previously, indicate that p21 inhibits two PCNA-dependent reactions, one leading to the repair of damaged DNA and the other leading to the elongation of primed DNA templates by pol δ and/or pol ε (Flores-Rozas *et al.*, 1994; Waga *et al.*, 1994; Li *et al.*, 1994). At present it is not clear whether it is pol δ and/or pol ε that participates in nucleotide excision repair (Zeng *et al.*, 1994; Aboussekhra *et al.*, 1995), and there is also some controversy concerning which of these two polymerases participates in lagging strand maturation and in leading strand synthesis during DNA replication (Morrison *et al.*, 1990; Burgers, 1991; Lee *et al.*, 1991b; Araki *et al.*, 1992; Budd and Campbell, 1993). Although it is difficult to compare the SV40 replication reaction and the nucleotide excision repair assay carried out by crude extracts, the results presented here indicate that there may be a quantitative difference in the inhibitory effects of p21 in these reactions (see Table I for a comparison). As expected from previous observations regarding the elongation of primed templates over short lengths (10 nucleotides) and over long regions (7 kilobases), the replication reaction is more sensitive to inhibition by p21 than the repair reaction (Flores-Rozas *et al.*, 1994). These studies carried out using the highly purified pol δ holoenzyme concluded that the molar ratio of p21 to PCNA (as a monomer) required to block short length extensions was nearly 100-fold greater than the molar ratio required for inhibition of elongation over a 7-kilobase length. From the measurements of PCNA present in crude extracts, a much lower ratio of p21 to PCNA inhibited the repair reactions (a molar ratio of p21 to PCNA of ~3, inhibited repair about 50%).

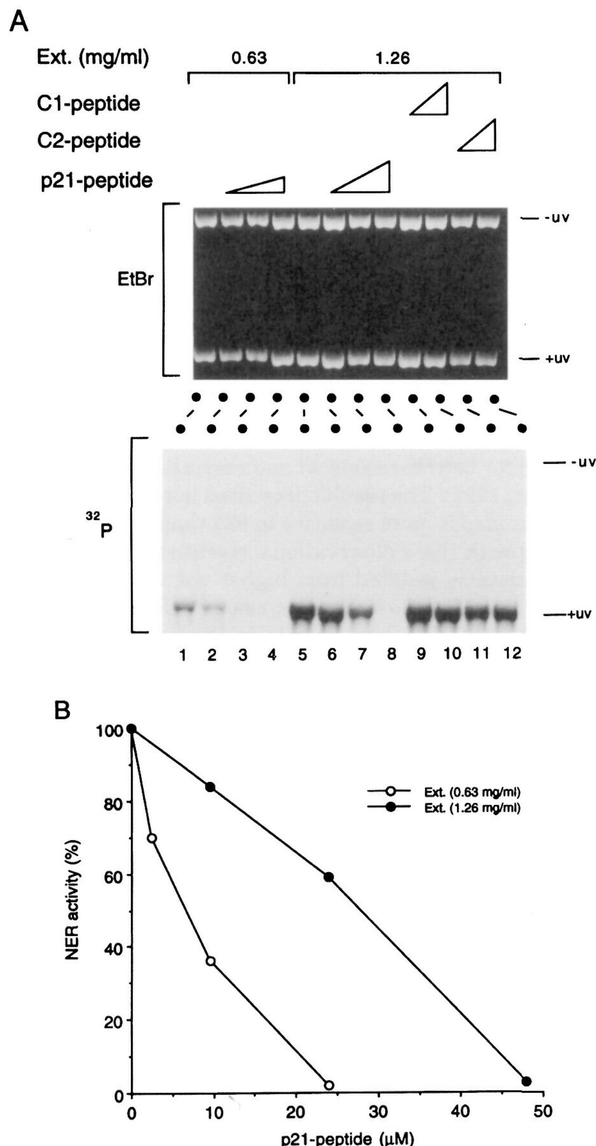

FIG. 5. **The p21 carboxyl-terminal peptide inhibits the repair of UV-damaged DNA.** *A*, reactions contained HeLa cytosolic extracts at 0.63 mg of protein/ml (*lanes 1–4*) or 1.26 mg of protein/ml (*lanes 5–12*) and other components, as described under "Materials and Methods." The p21 carboxyl-terminal peptide was added at 2.4 μM (*lane 2*), 9.6 μM (*lanes 3* and *6*), 24 μM (*lanes 4* and *7*), or 48 μM (*lane 8*). A control peptide C1 (NLFCSEEMPSSDDC) was added at 24 μM (*lane 9*) or 48 μM (*lane 10*). Another control peptide C2 (EPPLSQEAFADLWKK) was added to 24 μM (*lane 11*) or 48 μM (*lane 12*). After incubation at 37 °C for 60 min, reactions were stopped and analyzed as described under "Materials and Methods." Both the ethidium bromide staining and autoradiogram of the gel are shown. *B*, quantitation of the experiment shown in *A*. In the absence of the p21 peptide, 0.5 and 1.14 pmol of [^{32}P]dCMP were incorporated into the UV-damaged plasmid with 0.63 and 1.26 mg/ml HeLa extracts, respectively (each representing 100% activity).

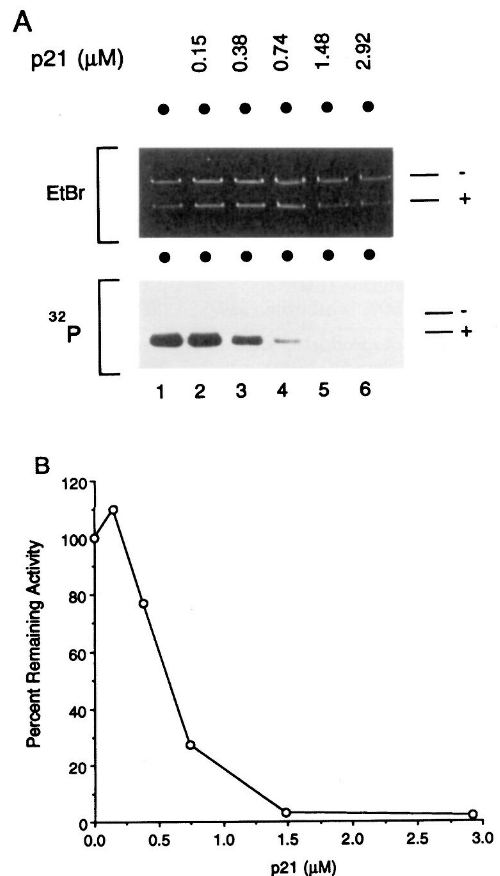

FIG. 6. **p21 inhibits repair of AAAF-treated DNA in WI-L2 whole cell extracts.** *A*, DNA repair reactions were carried out in the absence (*lane 1*) or in the presence of increasing amounts of p21 (*lanes 2–6*) under conditions described under "Materials and Methods." After incubation at 30 °C for 3 h, reactions were stopped and analyzed as described (Wood *et al.*, 1988). Both the ethidium bromide staining and autoradiogram of the gel are shown. *B*, quantitation of the results shown in *A*.

Since nucleotide excision repair requires relatively short-patch synthesis by pol δ or pol ϵ, we anticipated that this PCNA-dependent reaction would be as insensitive to p21 inhibition as that found in the elongation over a 10-nucleotide stretch. However, this was not the case, suggesting that the role of PCNA in nucleotide excision repair may prove more complex than the role played in the pol δ or pol ϵ holoenzyme reaction. At present, it is not clear how PCNA participates in the repair of damaged DNA. It is clear that RF-C is essential for the loading of PCNA in replication reactions, and an involvement of RF-C in the repair of damaged DNA has been suggested by Aboussekhra *et al.* (1995). In contrast to pol δ, pol ϵ can extend DNA chains in the absence of RF-C and PCNA (Nishida *et al.*, 1988; Burgers, 1991; Lee *et al.*, 1991b). However, in the presence of salt concentrations more closely resembling physiological conditions, pol ϵ activity becomes completely dependent on both RF-C and PCNA (Burgers, 1991; Lee *et al.*, 1991b). Although PCNA can be loaded onto primed DNA templates in the absence of RF-C, such reactions required extremely high levels of PCNA (Burgers and Yoder, 1993).

The results presented here are in contrast to the data presented by Li *et al.* (1994) and by Shivji *et al.* (1994), who have reported that high levels of p21 had no effect on the repair of UV damaged DNA. The levels of p21 used in their experiments (up to 40 μg/ml, equivalent to 1.9 μM), are well within the range that we found inhibitory. The reasons for the discrepancy are unclear. One contributing factor that we have noticed concerns the degree of aggregation of p21. We have observed that, with time, the inhibitory effects of p21 in both replication and repair decreased, and this was correlated with increased aggregation. This was observed with both free p21 and the histidine-tagged p21.

Li *et al.* (1994) have proposed that pol δ or pol ϵ acts to completely fill in the 30-nucleotide stretch essential for the repair of the excised damaged region of DNA. In their model, they proposed that PCNA, complexed with p21, is effective in fixing the polymerase to the primer end but PCNA needs not act as a processivity factor since the polymerase acts over a short region. This provocative model suggests that the p21-

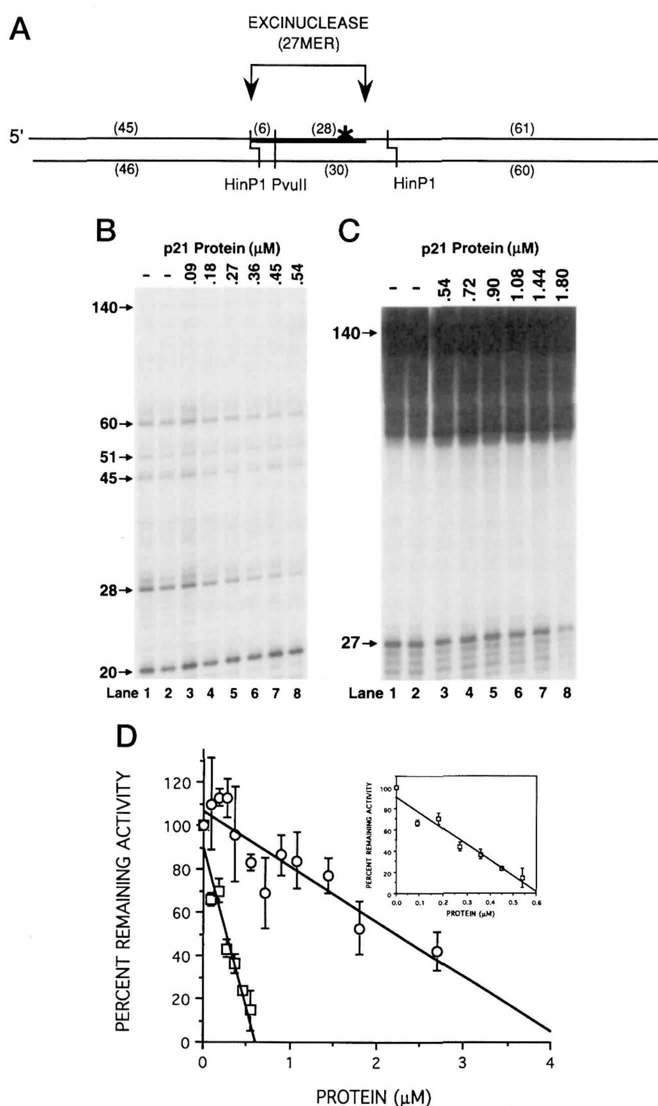

FIG. 7. **p21 protein inhibits both the excision and resynthesis steps of nucleotide excision repair.** *A*, schematic drawing of the substrate and relevant restriction enzyme sites used to examine excision and damage-specific DNA synthesis. *B* and *C*, effect of p21 on the repair synthesis (*B*) and excision activity (*C*). Assay conditions were as described under "Materials and Methods." *D*, the remaining activity (%) was determined relative to control reactions that lacked p21. The activities observed in the absence of the inhibitors represented 100% activity. The data points indicated represent the average of two to four experiments, and the standard error is shown. In the figure presented, the excision activity, assayed by release of radiolabeled fragments containing the damaged site, is represented by *circles*, whereas the repair synthesis, assayed by the incorporation of radiolabel into the specific restriction fragment containing the repaired region, is represented by *squares*.

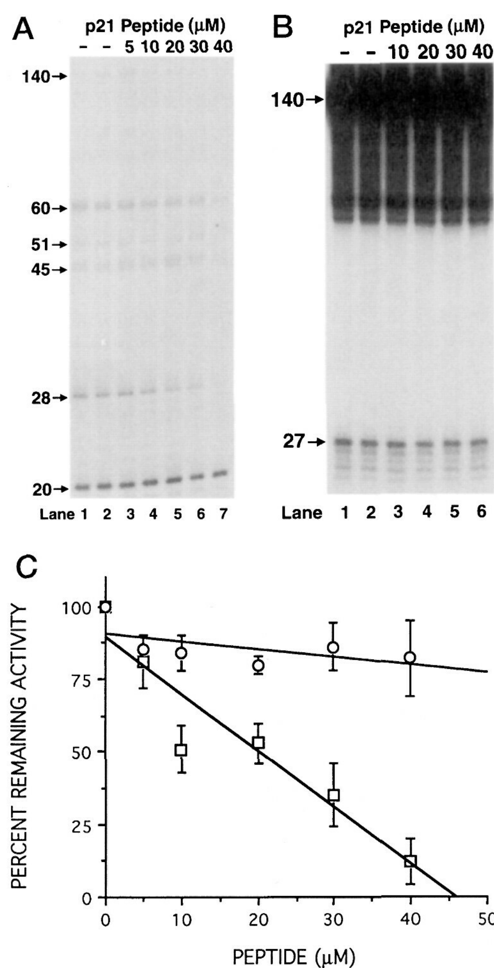

FIG. 8. **Effect of the p21 carboxyl-terminal peptide on the repair synthesis (*A*) and excision activity (*B*).** Assay conditions were as described under "Materials and Methods." *C*, the remaining activity (%) was determined relative to control reactions that lacked the p21 peptide. The activities observed in the absence of the inhibitors represented 100% activity. The data points indicated represent the average of two to four experiments, and the standard error is shown. In the figure presented, the excision activity was assayed by the release of radiolabeled fragments containing the damaged site and is represented by *circles*. The repair synthesis, assayed by the incorporation of radiolabel into the specific restriction fragment containing the repaired region, is represented by *squares*.

PCNA complex governs how the polymerase interacts with PCNA. In contrast, our findings suggest that the formation of the p21-PCNA complex, which requires high levels of p21, inhibits repair of damaged DNA. The availability of purified excision-repair systems (Mu *et al.*, 1995; Aboussekhra *et al.*, 1995) should permit a better evaluation of the role of PCNA and the effect of p21.

Chen *et al.* (1995) and Luo *et al.* (1995) have recently shown that while the NH_2-terminal domain of p21 contains the CDK inhibitory activity, the CH_2-terminal domain contains the PCNA binding and DNA synthesis inhibitory activities. Luo *et al.* (1995) have further demonstrated that each of these domains (codons 1–75 as the NH_2 terminus, and codons 76–164 as the carboxyl domain), when expressed in R-1B/L17 cells, was sufficient to block DNA synthesis, although the carboxyl domain of p21 was less efficient. There is some controversy concerning the growth-inhibitory effect of the carboxyl terminus of p21 *in vivo*. Chen *et al.* (1995) have reported that overexpression of the NH_2-terminal domain of p21 (codons 1–90), but not the carboxyl end (codons 87–164) in human SaOs2 cells, blocked cell growth. However, the expression of the carboxyl end of p21 and its interaction with PCNA in transfected SaOs2 cells was not examined.

It appears that CDKs are the prime targets of p21 in blocking entry into S phase. What is the physiological significance of inhibition of PCNA function by p21? We postulate that there is a quantitative difference in the level of p21 induced by p53 in response to DNA damage. Relatively low levels of p21 are sufficient to inhibit CDK activity but not to inhibit PCNA. As a result, cells are arrested in G_1 to repair DNA damage. However, when the level of p21 induced becomes higher, perhaps due to severe DNA damage, it acts to inhibit PCNA function in both DNA replication and repair. The PCNA-inhibitory function of p21 is perhaps related to p53-induced apoptosis. However, it remains to be determined whether p21 is required for

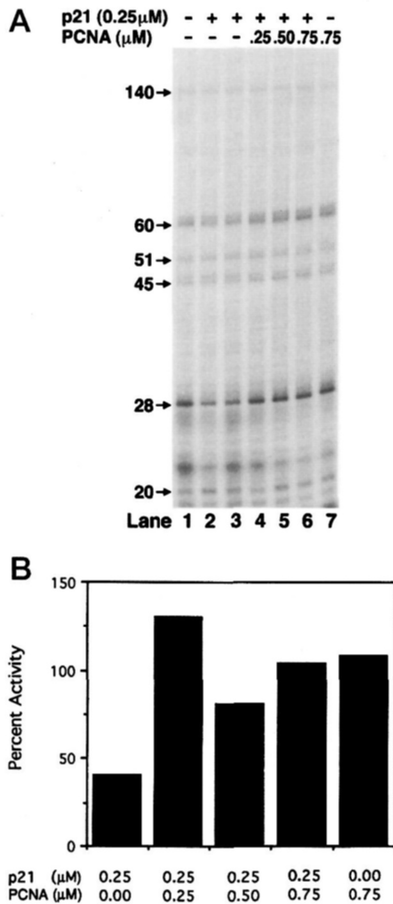

FIG. 9. **Influence of the addition of exogenous PCNA on the repair synthesis of the 140-mer oligonucleotide substrate carried out in the presence or absence of p21.** Assay conditions were as described under "Materials and Methods." *A*, an autoradiograph illustrating the repair synthesis assay with HeLa CFE, p21 and PCNA. *Numbers* to the *left* indicate the size in nucleotides of the DNA fragments. The 28-mer is the fragment containing the repair patch, whereas the 20-mer is the internal control DNA. The other radiolabeled fragments result from nonspecific incorporation. *B*, quantitation of the results shown in *A*. The value 100% (shown in *lane 1*) was obtained from the amount of radioactivity recovered in the 28-mer.

p53-mediated apoptosis and whether DNA replication and repair functions are impaired by p21 in cells committed to apoptosis.

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