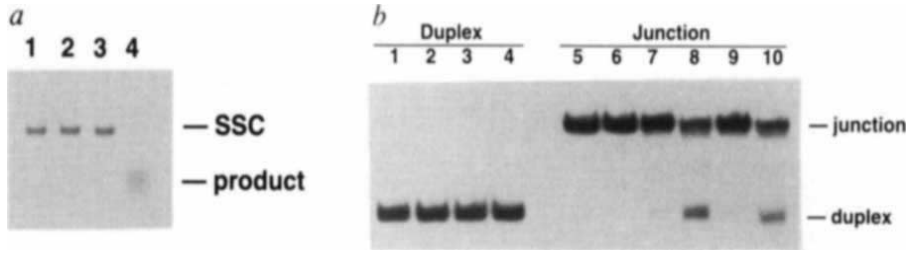


FIG. 4 Rad10 protein is required for cleavage of single-stranded (ss) DNA but has no effect on Holliday junction cleavage. *a*, Rad1–Rad10 nuclease action on ssDNA. Viral circular ssM13 DNA (200 ng, lanes 1–4) was incubated in buffer B (see Fig. 1 legend) with 150 ng Rad1 (lane 2), with 50 ng Rad10 (lane 3), or with 150 ng Rad1 and 50 ng Rad10 together (lane 4) for 20 min at 30 °C. Reaction mixtures were deproteinized and analysed in a 0.8% agarose gel as described[2]. *b*, Rad10 does not affect Holliday junction cleavage. Duplex DNA (lanes 1–4) and X12 junction DNA (lanes 5–10), 0.5 ng and 1 ng respectively, were incubated with RAD1 (lanes 3, 7, 8), with Rad10 (lanes 2, 6), or with both proteins together (lanes 4, 9, 10) for 15 min at 30 °C and analysed. In the last case (lanes 4, 9, 10), Rad1 and Rad10 were mixed together and incubated for 15 min on ice before incubation with DNA. Either 15 ng (lanes 7, 9) or 30 ng (lanes 3, 4, 8, 10) Rad1 and 10 ng of Rad10 (lanes 2, 4, 6, 9, 10) were used.

In *Escherichia coli*, the product of *RuvC* resolves the Holliday structure through DNA strand cleavage[7,9]. But whereas Rad1 incises at multiple sites in the junction DNA without any strong sequence preference, endonucleolytic scission of junction DNA by RuvC protein shows highly restricted specificity as it occurs efficiently only at the 3′ side of thymine residues[10]. Furthermore, RuvC introduces symmetrically related nicks in junction DNA[10], whereas Rad1 nicks junction DNA at multiple sites, many of which are asymmetrical.

Our finding that Rad1 cleaves Holliday junctions agrees with genetic studies indicating that the *RAD1* and *RAD10* counterparts in the fission yeast *Schizosaccharomyces pombe* function in the resolution of recombination intermediates during mating-type switching. Of the ten *swi* genes involved in mating-type switching in *S. pombe*, *swi4*, *swi8*, *swi9* and *swi10* affect the resolution of recombination intermediates, and because of aberrant resolution, mutants of these genes show increased incidence of rearrangements of the mating-type locus[11]. *swi9* and *swi10* are the respective *S. pombe* homologues of *S. cerevisiae RAD1* and *RAD10* genes[12,13]. Even though Rad1 alone cleaves Holliday junction, it introduces many asymmetrical nicks in the junction DNA. We suggest that a complex consisting of Rad1, Rad10 and the *S. cerevisiae* counterparts of the swi4 and swi8 proteins would resolve Holliday junction by nicking strands of the same polarity in a symmetric fashion. However, it is also possible that some of these other proteins function in other phases of recombination[5]. □

Received 2 June; accepted 17 August 1994.

1. Prakash, S., Sung, P. & Prakash, L. *A. Rev. Genet.* **27,** 33–70 (1993).
2. Sung, P., Reynolds, P., Prakash, L. & Prakash, S. *J. biol. Chem.* **268,** 26391–26399 (1993).
3. Tomkinson, A., Bardwell, A. J., Bardwell, L., Tappe, N. & Friedberg, E. C. *Nature* **362,** 860–862 (1993).
4. Bailly, V., Sommers, C. H., Sung, P., Prakash, L. & Prakash, S. *Proc. natn. Acad. Sci. U.S.A.* **89,** 8273–8277 (1992).
5. Sung, P., Prakash, L. & Prakash, S. *Nature* **355,** 743–745 (1992).
6. Taylor, A. F. *Cell* **69,** 1063–1065 (1992).
7. Dunderdale, H. J. *et al. Nature* **354,** 506–510 (1991).
8. Watkins, J. F., Sung, P., Prakash, L. & Prakash, S. *Molec. cell. Biol.* **13,** 7757–7765 (1993).
9. Iwasaki, H., Takahagi, M., Shiba, T., Nakata, A. & Shinagawa, H. *EMBO J.* **10,** 4381–4389 (1991).
10. Bennett, R. J., Dunderdale, H. J. & West, S. C. *Cell* **74,** 1021–1031 (1993).
11. Egel, R. in *Molecular Biology of the Fission Yeast* (eds Nasim, A., Young, P. & Johnson, B. F.) 31–73 (Academic, New York, 1989).
12. Carr, A. M. *et al. Molec. cell. Biol.* **14,** 2029–2040 (1994).
13. Rödel, C., Kirchhoff, S. & Schmidt, H. *Nucleic Acids Res.* **20,** 6347–6353 (1992).

ACKNOWLEDGEMENTS. This work was supported by the US National Cancer Institute and the Department of Energy.

Differential effects by the p21 CDK inhibitor on PCNA-dependent DNA replication and repair

Rong Li, Shou Waga, Gregory J. Hannon*, David Beach* & Bruce Stillman

Cold Spring Harbor Laboratory and * Howard Hughes Medical Institute, Cold Spring Harbor, New York 11724, USA

In mammalian cells, DNA damage increases the levels of the nuclear tumour-suppressor p53, resulting in elevated synthesis of p21, an inhibitor of cyclin-dependent kinases (CDK)[1–6]. p21 may also directly block DNA replication by inhibiting the proliferating-cell nuclear antigen (PCNA)[7], an essential DNA replication protein. However, PCNA is also required for nucleotide-excision repair of DNA[8], an intrinsic part of the cellular response to ultraviolet irradiation. Using an *in vitro* system[9], we now show that p21 does not block PCNA-dependent nucleotide-excision repair, in contrast to its inhibition of simian virus 40 DNA replication[7]. Furthermore, the short gap-filling DNA synthesis by PCNA-dependent DNA polymerases δ and ε is less sensitive to inhibition by p21 than is long primer-extension synthesis. The ability of p21 to inhibit the role of PCNA in DNA replication but not in DNA repair rationalizes *in vivo* data showing that genetic damage leads to inactivation of chromosomal replication while allowing damage-responsive repair.

To compare the effect of p21 on DNA repair and replication, whole-cell extracts were prepared from RKO cells, a human colorectal carcinoma cell line containing wild-type p53 (ref. 10). Following published procedures[9], we showed that the RKO cell extract preferentially repaired ultraviolet-damaged plasmid DNA templates in the presence of undamaged control DNA (Fig. 1*a*, lanes 1 and 2). The same extract also supports large-T-antigen-dependent replication of plasmids containing the simian virus 40 (SV40) replication origin (Fig. 1*a*, lanes 5 and 6). As shown previously[7], purified recombinant p21 abolished SV40 DNA replication *in vitro* (Fig. 1*a*, lanes 7 and 8; see Fig. 1*b* for quantification). Addition of purified PCNA overcame the p21 inhibition of DNA replication (ref. 7, and data not shown). In contrast, similar amounts of p21 did not affect DNA repair *in vitro* (Fig. 1*a*, lanes 3 and 4, and Fig. 1*b* for quantification). To verify that this result is not restricted to extracts from tumour cell lines, we prepared extracts from a cell line (GM02184) derived from healthy human lymphoblasts. As shown in Fig. 2, p21 again blocked SV40 DNA replication but was without effect on DNA repair. These *in vitro* results are consistent with data from ultraviolet-irradiated cells in which replicative DNA synthesis as well as cell-cycle progression is inhibited, yet DNA repair persists[11,12].

To ascertain the dependence of the *in vitro* repair system on PCNA, we fractionated the RKO cell extract so that reconstituted DNA repair depended on purified PCNA, replication protein A and a fractionated extract (fraction II) (Fig. 3, lanes 4 and 5; see also ref. 8). Replication protein A is a cellular single-

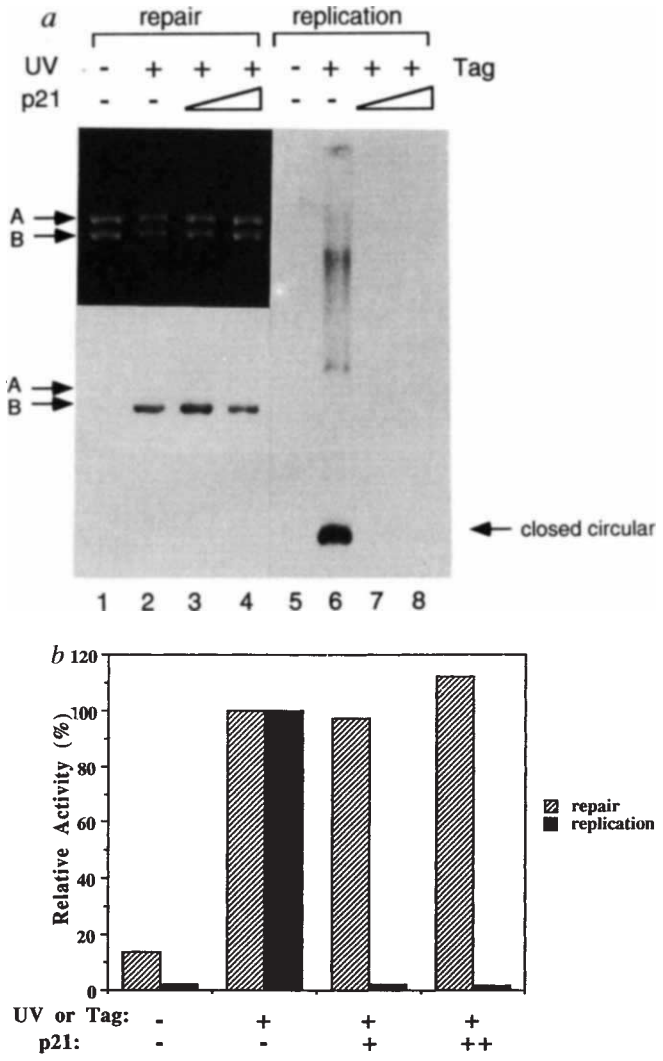


FIG. 1 p21 inhibits SV40 DNA replication, but not DNA repair. *a*, DNA repair (lanes 1–4) and replication (lanes 5–8) in an RKO cell extract. The top and bottom panels in lanes 1–4 show a photograph and an autoradiogram of the same gel. Arrows A and B represent pKSO (ref. 26), a non-irradiated plasmid and pSV011 (ref. 27), a UV-irradiated plasmid. Neither plasmid in lane 1 was irradiated. DNA replication was carried out in either the absence (lane 5) or presence of the SV40 large T-antigen (lanes 6–8). Replication and linearized repair products were analysed by agarose gel electrophoresis in the presence of ethidium bromide. The concentrations of p21 were 20 μg ml^{-1} (lanes 3 and 7) and 40 μg ml^{-1} (lanes 4 and 8). *b*, The autoradiogram was quantified by a Fuji Bio Imaging Analyzer. The repair (▨) and replication (■) signals in the absence of p21 (lanes 2 and 6 of *a*) are designated as 100%. The UV-specific repair synthesis was normalized against the background synthesis of the non-irradiated plasmid.

METHODS. RKO cell extract was prepared as described[26]. The protein concentration of the extract was 5–10 mg ml^{-1}. The SV40 DNA replication and *in vitro* repair reactions were done as described[9,27]. A typical replication and repair reaction contained 2 mg ml^{-1} of extract protein. A repair reaction contained 10 μg ml^{-1} each of the non-irradiated and UV-irradiated plasmids (at a UV dosage of 450 J m^{-2}). The crude extract and p21 were mixed and preincubated on ice for 30 min; DNA templates were added and the reaction was further incubated for 2 h at either 30 °C (repair) or 37 °C (replication). Histidine-tagged p21 was expressed and purified as described[7]. The DNA templates were prepared by a Qiagen plasmid kit and sucrose gradient centrifugation[9].

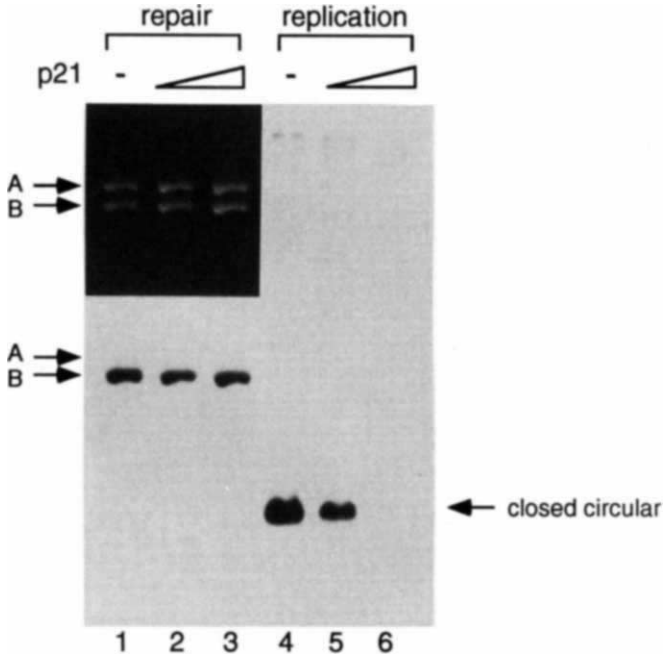


FIG. 2 Effects of p21 on DNA repair and DNA replication in a whole cell extract from a healthy human lymphoblast. *In vitro* repair (lanes 1–3) and SV40 DNA replication (lanes 4–6) were carried out essentially as described for Fig. 1. Also shown in lanes 1–3 is a photograph (top) of the same ethidium-bromide-stained gel. The two concentrations of p21 used in this experiment were 2 μg ml^{-1} (lanes 2 and 5) and 5 μg ml^{-1} (lanes 3 and 6). The human lymphoblast cell line GM02184 (purchased from NIGMS human genetic mutant cell repository at Camden, NJ) were grown under the conditions given by the supplier. Whole-cell extract was prepared following the protocol described previously[26].

stranded DNA-binding protein required for both SV40 DNA replication[13] and nucleotide-excision repair[14]. We tested the effect of p21 on DNA repair using known amounts of both PCNA and p21 in this partially reconstituted system. The concentration of purified p21 (monomer) reached a 240-fold (Fig. 3, lane 7) or 60-fold (lane 8) molar excess over PCNA (trimer), but the *in vitro* repair was not significantly affected (Fig. 3: compare lanes 4 and 5 with 7 and 8). SV40 DNA replication requires much more PCNA than repair of ultraviolet-damaged DNA *in vitro*[8,15], yet repair is refractory to p21 whereas replication is p21-sensitive. Taken together, these data suggest that p21 can distinguish between the activities of PCNA in DNA replication and those in DNA repair.

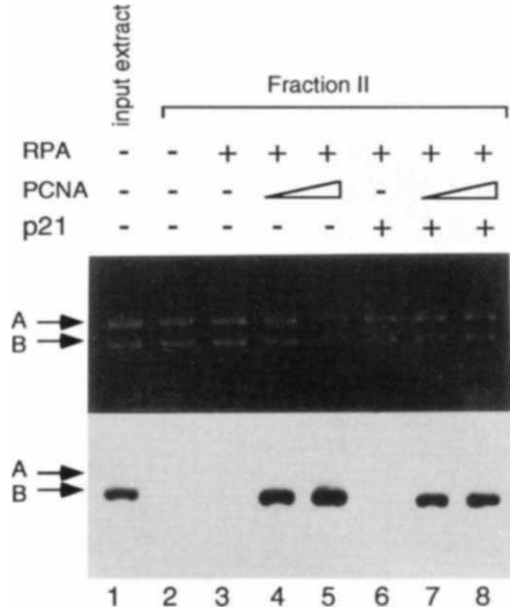
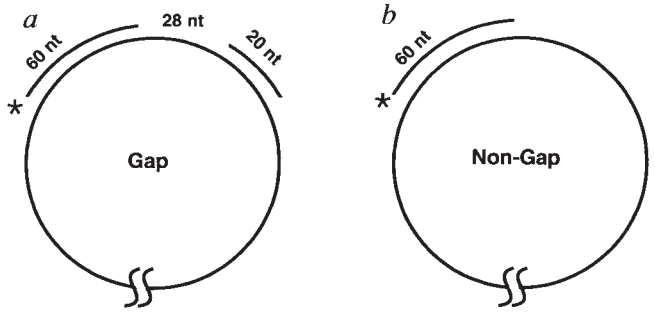


FIG. 3 Characterization of the effect of p21 in a reconstituted PCNA-dependent repair assay. *In vitro* repair reactions were carried out in either the crude RKO cell extract (lane 1) or a fractionated extract (fraction II; lanes 2–8) in the presence or absence of 6 μg ml^{-1} of RPA, and in the presence or absence of PCNA (0.1 μg ml^{-1} for lanes 4 and 7 or 0.4 μg ml^{-1} for lanes 5 and 8). The concentration of p21 used in lanes 6–8 was 5 μg ml^{-1}. Plasmids A (non-irradiated) and B (UV-irradiated) were the same as those used in Figs 1 and 2. The top panel shows a photograph of the ethidium-bromide-stained gel, and the lower panel represents an autoradiogram of the same gel.

METHODS. The whole-cell extract from RKO cells was fractionated on a phosphocellulose column as previously described[28], with some minor modifications. Essentially, the extract was adjusted to 0.2 M NaCl and applied to the column. The flow-through (fraction I) contained all the replication protein A (RPA) and most of the PCNA from the input extract. The high-salt eluate from 0.2 to 1 M NaCl (fraction II) contained all the other repair proteins. The residual small amount of PCNA retained in fraction II was further removed by an anti-PCNA monoclonal antibody (PC10) coupled to protein A–Sepharose beads. The resulting fraction was completely depleted of both RPA and PCNA as judged by ECL western blot detection (Amersham) and the *in vitro* repair assay. RPA used here was purified from human 293 cells[27].



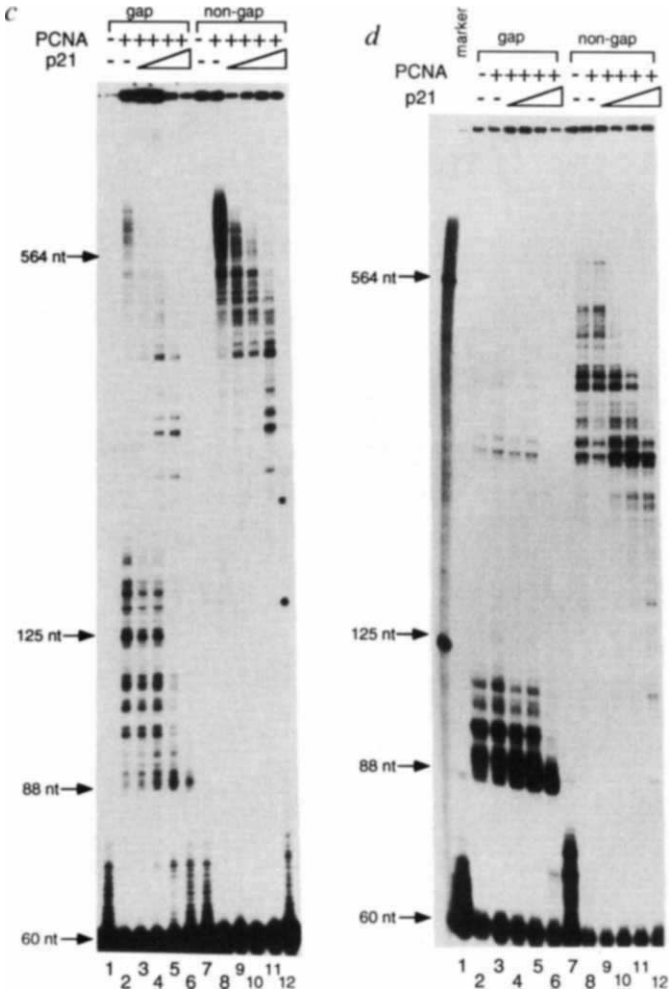


FIG. 4 p21 preferentially inhibits synthesis of long DNA fragments by polymerases δ and ε. *a*, *b*, Schematic diagrams of the DNA templates. The numbers indicate the lengths (in nucleotides) of the two primers and the single-stranded gap between them. The star represents the radioactive labelled end. *c*, The polymerase δ assay was carried out using either a gapped template (lanes 1–6) or a non-gapped template (lanes 7–12). The final concentrations of p21 used in lanes 3–6 and 9–12 were 2, 4, 8 and 16 μg ml^{-1}, respectively. The positions of different lengths of DNA fragments are indicated in nucleotides (nt). The position of 60 nt is the 3′ end of the labelled primer. *d*, Same as *c*, but DNA synthesis by polymerase ε.

METHODS. A primer of nucleotides 928–987 in pBluescript SK(−) (Stratagene) was labelled and annealed to the single-stranded form of pBluescript SK(−) either alone (non-gapped) or with a primer of nucleotides 880–899 (gapped). The labelled and unlabelled primers were in 2- and 10-fold molar excess over the single-stranded DNA template, respectively. The polymerase assays were carried out in 40 mM Tris, pH 7.8, 1 mM dithiothreitol, 100 μg ml^{-1} BSA, 7 mM $MgCl_2$, 2 mM ATP, 120 μM dNTP, 4 μg ml^{-1} DNA template, 40 μg ml^{-1} RPA, 36 μg ml^{-1} replication factor C (RFC; fraction IV), and when indicated, 4 μg ml^{-1} PCNA, 1 unit of polymerase ε and 0.1 units polymerase δ per 10-μl reaction (for unit definition, see refs 27, 29). For PCNA-dependent synthesis by polymerase ε, NaCl was added to a final concentration of 125 mM. The amounts of the two polymerases were chosen to generate approximately an equivalent amount of DNA synthesis on the primed-ssDNA templates. Calf polymerase δ and human RFC were purified as described[27]. Calf polymerase ε was a gift from U. Hubscher at University of Zurich-Irchel, Switzerland[29]. p21 was preincubated on ice for 30 min with the rest of the reaction mixture minus the DNA substrate. DNA substrate was added and the reaction was further incubated at 37 °C for 1 h. The reaction products were analysed on a 6% denaturing acrylamide gel.

Nucleotide-excision repair in mammalian cells can be separated into two steps[16]. First, the damaged DNA site is recognized and a fragment of 27–29 nucleotides containing the pyrimidine dimer is released by an incision/excision step, then PCNA-dependent repair synthesis closes the gap. In contrast, replicative DNA synthesis extends over much longer regions of DNA. It seemed plausible that the structural differences in DNA templates could lead to the differential effects of p21. Alternatively, a PCNA-dependent DNA polymerase other than polymerase δ, such as polymerase ε, could be responsible for repair synthesis and be more resistant to p21 inhibition. For example, polymerase ε requires less PCNA than polymerase δ for polymerase activity[17].

To test these models, we designed two DNA templates mimicking those involved in either DNA repair or replication. The first template contains two oligonucleotides annealed to a circular, single-stranded DNA, creating a 28-nucleotide gap (Fig. 4*a*). In this case, the upstream primer is labelled and the downstream primer serves as a 'terminator' that will retard progress of the DNA polymerase. The second template is a primed single-stranded DNA without the second oligonucleotide, thus resembling a template for DNA replication (Fig. 4*b*). Synthesis by polymerase δ or ε was carried out in the presence of replication protein A, replication factor C (a DNA-polymerase accessory factor) and PCNA (where indicated).

As shown previously[17–20], polymerase δ activity was dependent on the presence of PCNA (Fig. 4*c*, compare lanes 1 and 7 with 2 and 8). In the case of the gapped template, the polymerase paused at multiple sites near the 3′ end of the gap sequence (88 nucleotides); in addition, there was a considerable amount of strand displacement synthesis beyond the gap. Synthesis of long DNA products (for example, above the 564-nucleotide marker) on both templates was greatly diminished by increasing amounts of p21 (Fig. 4*c*, lanes 3–6, 9–12; the molar ratio of p21 to PCNA in lanes 6 and 12 was 20 to 1). Alkaline agarose gel electrophoresis confirmed that synthesis of larger DNA products (500–2,000 nucleotides) was greatly reduced by p21 (data not shown). In contrast, PCNA-dependent gap-filling DNA synthesis was relatively unaffected (Fig. 4*c*, lanes 3–6; 88 nucleotides). The effect of p21 on polymerase ε activity was assessed using conditions under which the polymerase activity was PCNA-dependent (Fig. 4*d*). Again, p21 blocked synthesis of long DNA fragments (Fig. 4*d*, lanes 9–12; around the 564-nucleotide marker), but not the short patch synthesis by polymerase ε (Fig. 4*d*, lanes 3–6; 88 nucleotides). Compared with polymerase δ, synthesis by polymerase ε on the gapped templates was more confined to the gap region (Fig. 4*d*, lanes 1–6), and its PCNA-dependent activity seemed to be more resistant to p21, suggesting that it was a better polymerase for DNA repair synthesis (for example, compare lanes 8 and 9 in Fig. 4*c* with those in Fig. 4*d*). Notably, both polymerases have been implicated in DNA repair[21–23]. Although our results do not address which polymerase plays a major role in nucleotide-excision repair *in vivo*, they show that short-gap synthesis by either polymerase is relatively insensitive to inhibition by p21.

PCNA has a dual function in SV40 DNA synthesis[13]. It recognizes a primer–template junction in cooperation with replication factor C and facilitates loading of polymerase δ; PCNA also enhances the processivity of polymerase δ during the elongation step. We suggest that the role of PCNA in processivity may be more sensitive to p21 than is its role in polymerase loading, and the PCNA–p21 interaction may arrest the polymerase and cause it to fall off the template. In repair synthesis, it is conceivable that the primary role of PCNA is to help the polymerase (δ or ε) localize to the junction of the incised DNA. Once bound to the template, the polymerase may be able to synthesize the short patch of DNA without the aid of PCNA. In addition, other repair-specific proteins[24,25] may further contribute to immunizing the repair machinery to p21.

DNA damage poses a tremendous challenge to the integrity of the genome. The post-damage events in normal cells ensure that any errors caused by the damage are corrected before propagation of the chromosomes. Based on our current understanding, elevated p21 levels block cell-cycle progression and DNA replication by coordinately inhibiting the functions of both CDKs and PCNA. The results presented here suggest a selectivity in p21 function which allows it to arrest DNA replication while permitting active DNA repair. p21 and its interaction with CDK, cyclin and PCNA may also be involved in normal proliferating cells. It is possible that p21 interaction with PCNA couples DNA repair to DNA replication during S phase and if the link between p21 and PCNA is disrupted, post-replicative repair could be abrogated, leading to genetic damage. We suggest that p21, PCNA and its partner, replication factor C might function, perhaps in cooperation with cyclin and CDK, to coordinate the replication and repair machineries with cell-cycle progression in both normal and damaged cells. □

Received 13 July; accepted 18 August 1994.

1. El-Deiry, W. S. *et al. Cell* **75,** 817–825 (1993).
2. Gu, Y., Turck, C. W. & Morgan, D. O. *Nature* **366,** 707–710 (1993).
3. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K. & Elledge, S. J. *Cell* **75,** 805–816 (1993).
4. Xiong, Y. *et al. Nature* **366,** 701–704 (1993).
5. Xiong, Y., Zhang, H. & Beach, D. *Genes Dev.* **7,** 1572–1583 (1993).
6. Dulic, V. *et al. Cell* **76,** 1013–1023 (1994).
7. Waga, S., Hannon, G. J., Beach, D. & Stillman, B. *Nature* **369,** 574–578 (1994).
8. Shivji, M. K. K., Kenny, M. K. & Wood, R. D. *Cell* **69,** 367–374 (1992).
9. Wood, R. D., Robbins, P. & Lindahl, T. *Cell* **53,** 97–106 (1988).
10. Kastan, M. *et al. Cell* **71,** 587–597 (1992).
11. Friedberg, E. C. *DNA Repair* (Freeman, San Francisco, 1985).
12. Lu, X. & Lane, D. P. *Cell* **75,** 765–778 (1993).
13. Melendy, T. & Stillman, B. in *Nucleic Acids and Molecular Biology* Vol. 6 (eds Eckstein, F. & Lilly, D. M. J.) 129–158 (Springer, Berlin and Heidelberg, 1992).
14. Coverley, D. *et al. Nature* **349,** 538–541 (1991).
15. Tsurimoto, T. & Stillman, B. *J. biol. Chem.* **266,** 1961–1968 (1991).
16. Svoboda, D. L., Taylor, J. S., Hearst, J. E. & Sancar, S. *J. biol. Chem.* **268,** 1931–1936 (1993).
17. Podust, V. N., Georgaki, A., Strack, B. & Hubscher, U. *Nucleic Acids Res.* **20,** 4159–4165 (1992).
18. Bravo, R., Frank, R., Blundell, P. A. & MacDonald-Bravo, H. *Nature* **326,** 515–517 (1987).
19. Prelich, G. *et al. Nature* **326,** 517–520 (1987).
20. Lee, S.-H., Zhen-Qiang, P., Kwong, A. D., Burgers, P. M. J. & Hurwitz, J. *J. biol. Chem.* **266** 22707–22717 (1991).
21. Nishida, C., Reinhard, P. & Linn, S. *J. biol. Chem.* **263,** 501–510 (1988).
22. Wang, Z.-G., Wu, X.-H. & Friedberg, E. C. *Molec. cell. Biol.* **13,** 1051–1058 (1993).
23. Zeng, X.-R., Jiang, Y., Zhang, S.-J., Hao, H. & Lee, M. Y. W. T. *J. biol. Chem.* **269,** 13748–13751 (1994).
24. Hoeijmakers, J. H. J. *Trends Genet.* **9,** 211–217 (1993).
25. Aboussekhra, A. & Wood, R. D. *Curr. Opin. Genet. Dev.* **4,** 212–220 (1994).
26. Yang, L., Li, R., Mohr, I. J., Clark, R. & Botchan, M. R. *Nature* **353,** 628–632 (1991).
27. Waga, S., Bauer, G. & Stillman, B. *J. biol. Chem.* **269,** 10923–10934 (1994).
28. Prelich, G. & Stillman, B. *Cell* **53,** 117–126 (1988).
29. Weiser, T., Gassmann, M., Thommes, P., Ferrari, E. & Hafkemeyer, P. *J. biol. Chem.* **266,** 10420–10428 (1991).

ACKNOWLEDGEMENTS. We thank U. Hübscher for purified calf thymus DNA polymerase ε, M. Kastan for the RKO cell line, and S. Bell, W. Herr, P. Kaufman, T. Melendy and A. Stenlund for critical reading of the manuscript. This research was supported by a NIH grant to B.S. R.L. is a special fellow of the Leukemia Society of America. G.H. is a fellow of the Damon Runyon–Walter Winchell Cancer Research Fund. D.B. is an Investigator of the Howard Hughes Medical Institute.

ERRATUM

Mice with targeted disruptions in the paralogous genes *hoxa-3* and *hoxd-3* reveal synergistic interactions

Brian G. Condie & Mario R. Capecchi

Nature **370,** 304–307 (1994)

FIGURES 1 and 2 in this Letter were accidentally transposed during the production process. The legends are correct as published. □