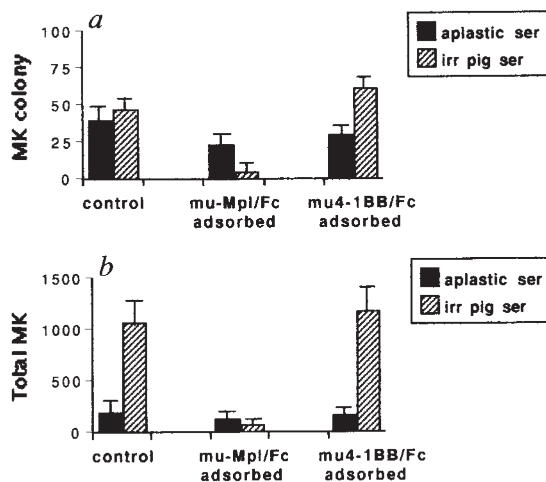

FIG. 4 Effect of serum from a post-irradiated pig on human CFU-MK colony formation. Mononuclear human bone marrow cells were seeded at a concentration of 2×10^5 cells per ml in plasma clot cultures in the presence of human aplastic serum or post-irradiated pig serum, untreated or pre-adsorbed on fusion proteins. On day 11 of culture, CFU-MK-derived colonies were visualized by an immunochemical reaction as described[7]. *a*, MK colonies numbers in each culture conditions (mean ± s.d. of 3 experiments in duplicate). *b*, Absolute numbers of MK in a total of 12 culture dishes (mean ± s.d.).

anti-CD61 monoclonal antibody, stained with propidium iodide and analysed by flow cytometry. The mean absolute number of MK found in cultures with untreated or mu-Mpl/Fc-adsorbed serum was 16,400 and 1,900 per 1×10^6 cells, respectively (4 replicate experiments). However, there was no noticeable difference in the modal ploidy distribution because only rare MK (<1%) reached a ploidy level above 16*N*, as previously reported[14] (data not shown). Thus, on purified CD34-positive cells, Mpl-L in irradiated pig serum appears to act mainly as a potent MK proliferation factor.

In summary, we have demonstrated that the recombinant Mpl-R directly binds a ligand that is present in sera from irradiated animals and transmits a mitogenic signal. However, this observation does not exclude the possibility that other subunits might be needed for full function of Mpl-R as has been demonstrated for other haematopoietin receptors[15,16]. The data provide evidence that Mpl-L is a humoral factor that induces proliferation and differentiation of megakaryocyte progenitors and strongly suggest that Mpl-L plays a pivotal role in regulating platelet homeostasis. The full spectrum of activities of Mpl-L will be precisely defined when the recombinant molecule becomes available and can be used in single cell assays. ☐

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1. Williams, N., Eger, R. R., Jackson, H. M. & Nelson, D. J. *J. Cell Physiol.* **110,** 101–104 (1982).
2. Hoffman, R. *Blood* **74,** 1196–1212 (1989).
3. McDonald, T. P. *Expl Hematol.* **16,** 201–205 (1988).
4. Skoda, R. C. *et al. EMBO J.* **12,** 2645–2653 (1993).
5. Vigon, I. *et al. Proc. natn. Acad. Sci. U.S.A.* **89,** 5640–5644 (1992).
6. Vigon, I. *et al. Oncogene* **8,** 2607–2615 (1993).
7. Methia, N., Louache, F., Vainchenker, W. & Wendling, F. *Blood* **82,** 1395–1401 (1993).
8. Palacios, R. & Steinmetz, M. *Cell* **41,** 727–734 (1985).
9. Mazur, E. M. *et al. Blood* **76,** 290–297 (1990).
10. Hoffman, R., Yang, H. H., Bruno, E. & Straneva, J. E. *J. clin. Invest.* **75,** 1174–1182 (1985).
11. McDonald, T. P. *Proc. Soc. exp. Biol. Med.* **144,** 1006–1011 (1973).
12. Metcalf, D. In *The Hematopoietic Colony Stimulating Factors* 124–130 (Elsevier Science, Amsterdam, New York, Oxford, 1984).
13. Mazur, E. M. & South, K. *Expl Hematol.* **13,** 1164–1172 (1985).
14. Debili, N. *et al. Blood* **82,** 84–95 (1993).
15. Russell, S. M. *et al. Science* **262,** 1880–1883 (1993).
16. Kondo, M. *et al. Science* **262,** 1874–1877 (1993).
17. Namen, A. E. *et al. Nature* **333,** 571–573 (1988).
18. Hopp, T. P. *et al. Biotechnology* **6,** 1204–1210 (1988).
19. Goodwin, R. G. *et al. Eur. J. Immun.* **23,** 2631–2641 (1993).
20. Ziegler, S. F. *et al. New Biol.* **3,** 1242–1248 (1991).
21. Brizzard, B. L., Chubet, R. G. & Vizard, D. L. *Biotechniques* **16,** 730–734 (1994).
22. McMahan, C. J. *et al. EMBO J.* **10,** 2821–2832 (1991).
23. Fanslow, W. C., Anderson, D. M., Grabstein, K. H., Clark, E. A. & Cosman, D. *J. Immun.* **149,** 655–660 (1992).

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The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA

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THE p53 tumour-suppressor protein controls the expression of a gene encoding the p21 cyclin-dependent protein kinase (CDK) regulator[1–6]. Levels of p21 protein are increased in senescent cells and p21 overexpression blocks the growth of tumour cells[1,3,7]. In normal human cells, but not in many tumour cells, p21 exists in a quaternary complex with a cyclin, a CDK, and the proliferating-cell nuclear antigen (PCNA)[5,8]. p21 controls CDK activity, thereby affecting cell-cycle control[2–4,6], whereas PCNA functions in both DNA replication[9–12] and repair[13]. Here we use simian virus 40 DNA replication *in vitro* to show that p21 directly inhibits PCNA-dependent DNA replication in the absence of a cyclin/CDK. Furthermore, p21 blocks the ability of PCNA to activate DNA polymerase δ, the principal replicative DNA polymerase. This regulation results from a direct interaction between p21 and PCNA. Thus, during p53-mediated suppression of cell proliferation, p21 and PCNA may be important for coordinating cell-cycle progression, DNA replication and repair of damaged DNA.

To understand how p21 suppresses cell proliferation, we investigated whether p21 would affect SV40 DNA replication *in vitro* (reviewed in ref. 14–16). This cell-free system models eukaryotic DNA replication (reviewed in refs 14–17) and enzymatic synthesis of DNA dependent on the SV40 *ori* has been reconstituted using SV40 T-antigen and human DNA replication proteins[12,18]. We expressed histidine-tagged p21 (His-p21; Fig. 1*a*) and p21 fused to glutathione *S*-transferase (GST) (GST-p21) (data not shown) in *Escherichia coli*. Both purified forms of p21 inhibited CDK (data not shown) and DNA replication from the SV40 *ori* in a crude extract from human 293 cells[19] (Fig. 1*b*, and data not shown). This latter inhibition was dose-dependent and incomplete, even with large amounts of protein. Heat-treated His-p21, which still functions as a CDK inhibitor[6], could also inhibit DNA replication, arguing that inhibition was not due to a contaminating nuclease or other enzyme.

Circular DNA topoisomers (the final replication product) and DNA replication intermediates (which migrate more slowly during electrophoresis) disappeared as His-p21 was increased (Fig. 1*c*). Saturating amounts of His-p21 gave rise to a smear on the gel corresponding to a size of ~2.3 kilobases, which represents accumulation of early DNA-replication intermediates[10,11] (Fig. 1*c*, lanes 5 and 6). This indicated that p21 could be inhibiting elongation but not initiation of DNA replication. A specific inhibitor, p16, of the cyclin D-dependent kinase CDK4 (ref. 20), did not prevent DNA replication *in vitro*, so this effect must be

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specific to p21; moreover, it was also prevented by GST-p21 but not by unrelated GST-fusion proteins (data not shown).

To identify the specific target for p21-mediated inhibition of DNA replication, purified replication proteins were added to the reactions to determine whether they could counteract the inhibition by His-p21. Of these proteins, only PCNA could restore DNA replication in a dose-dependent manner (Fig. 2*a*). Neither DNA polymerase δ (pol δ) nor replication factor C (reviewed in refs 15, 16) altered the His-p21 effect (Fig. 2*a*), even though enough of each protein was used to support SV40 DNA replication (see later). When PCNA was added to reactions containing His-p21, circular DNA topoisomers were recovered (Fig. 2*b*, lanes 3–6), indicating that replication was fully restored. Replication protein A (refs 15, 16) stimulated DNA synthesis

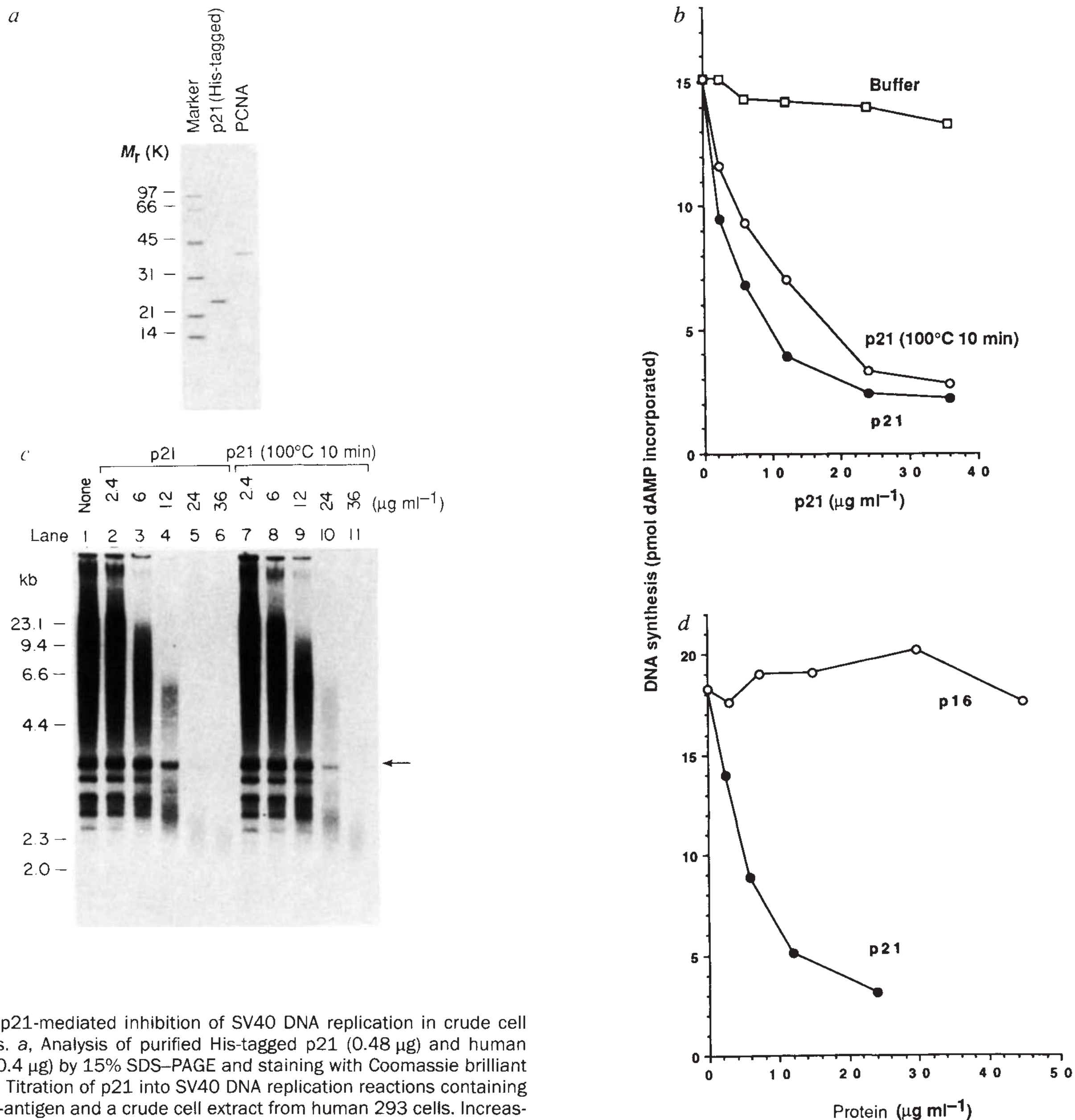

FIG. 1 p21-mediated inhibition of SV40 DNA replication in crude cell extracts. *a*, Analysis of purified His-tagged p21 (0.48 μg) and human PCNA (0.4 μg) by 15% SDS–PAGE and staining with Coomassie brilliant blue. *b*, Titration of p21 into SV40 DNA replication reactions containing SV40 T-antigen and a crude cell extract from human 293 cells. Increasing amounts of untreated (●) or heat-treated (100 °C, 10 min) (○) His-p21 were added to replication reactions, using an equivalent amount of buffer as a control (□). The amount of DNA synthesis is expressed as picomoles of dAMP incorporated in a 50-μl reaction in 1 hour. *c*, Analysis by electrophoresis on 1% agarose gels and autoradiography of products purified from each reaction in *b*. Lane 1, buffer control of same volume as p21 in lanes 6 and 11. Arrow indicates form-I relaxed DNA; other bands are form-I topoisomers. *d*, Comparative effects on DNA replication of His-p21 and His-p16 CDK inhibitors: increasing protein was added to replication reactions reconstituted with crude cell extract from human 293 cells.

METHODS. DNA was replicated from the SV40 origin in 10 μl containing 6 μg ml^{-1} pSV011 plasmid DNA[10], 44 μg ml^{-1} SV40 T-antigen, 1.5 mg ml^{-1} protein of a crude S100 cell extract from 293 cells as described[19]. Crude extracts were preincubated on ice for 30 min with His-p21 or His-p16 without SV40 T-antigen or plasmid DNA, then incubated at 37 °C for 1 h with T-antigen and DNA template. Measurement of incorporated radioactivity and the analysis of replication products have been described[18,29]. His-p21 contained an insertion (Gly-His_6) between amino acids 1 and 2 of the p21 sequence. It was expressed in bacteria and purified on fast-flow S–Sepharose (0–1 M NaCl gradient in 20 mM Tris, pH 8.0). p21-containing fractions were diluted 3-fold with 20 mM Tris, pH 8.0, 10% glycerol, and passed through fast-flow Q–Sepharose. Flow-through material was loaded onto Ni-NTA agarose (Qiagen) and His-p21 was eluted with 100 mM imidazole in 20 mM Tris, pH 8.0. Baculovirus-expressed, His-tagged p16 was provided by M. Serrano and D.B. (ref. 20). Human PCNA was expressed in *E. coli* and purified as before[18]; the other DNA replication proteins have been described[12,18,20,29].

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in the presence of His-p21, but only early intermediates were formed (S.W. and B.S., unpublished results). These results indicated that p21 was interfering with PCNA function during DNA replication. The human cell crude extract contained cyclin–CDK activity and therefore, it was possible that the p21 inhibition of DNA replication was indirect and involved its CDK inhibitory activity.

This possibility was investigated by examining the effect of p21 on SV40 *ori*-dependent DNA replication reconstituted with purified replication proteins, which did not include cyclins and CDKs, instead of the crude cell extract. Coordinated replication of both lagging and leading DNA strands occurs in this system[11,12]. We found that His-p21 again inhibited DNA replication (Fig. 3*a*) and that this inhibition could be reversed by the addition of excess PCNA (Fig. 3*b*). These results indicate that p21 and PCNA interact with one another directly, which was confirmed by analysis of DNA polymerase δ-directed DNA synthesis using an oligo(dT)/poly(dA)primer–template DNA. DNA polymerase δ needs PCNA for processive polymerase activity[9,21], and both are required for replication of the lagging and leading DNA strands[10-12]. Purified PCNA stimulated DNA synthesis by purified pol δ more than 10-fold in the absence of p21, but this was dramatically suppressed by His-p21 (Fig. 3*c*) and by GST-p21 (data not shown). Even with saturating amounts of His-p21, however, DNA synthesis by pol δ did not fall below the level achieved without PCNA, suggesting that p21 does not affect pol δ itself. An excess of PCNA, but not of pol δ, reversed this inhibition (Fig. 3*d*).

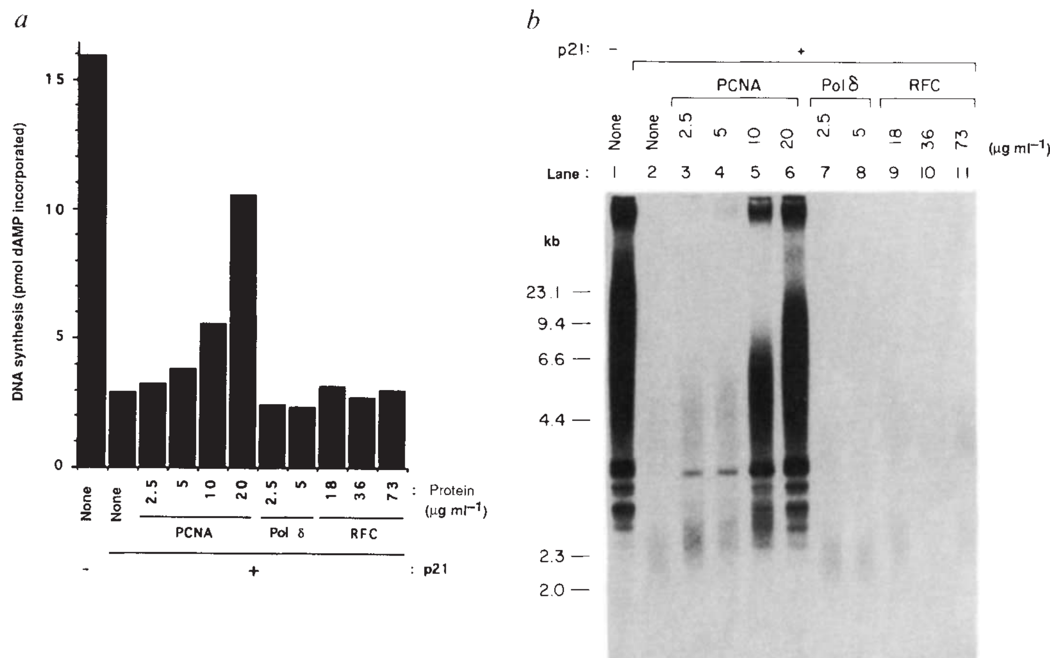

FIG. 2 Restoration of DNA replication by addition of PCNA. Crude cell extract from 293 cells (1.5 mg ml^{-1}) and 24 μg ml^{-1} His-p21 were preincubated on ice with various amounts of purified PCNA (Fig. 1*a*), pol δ or replication factor C (RFC) and replication assayed as for Fig. 1. *a*, Amount of DNA synthesis, as measured by pmol dAMP incorporated per hour, and *b*, analysis of purified products by 1% agarose gel electrophoresis.

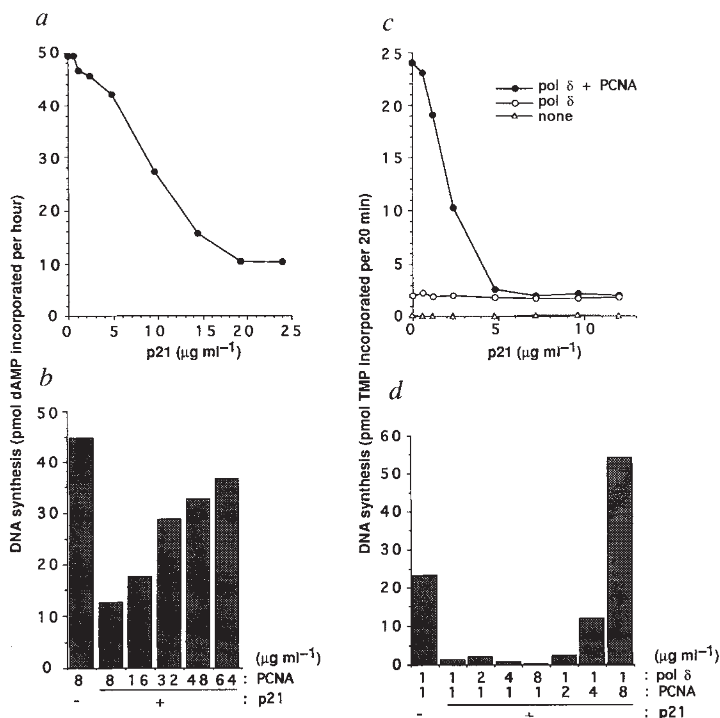

FIG. 3 Inhibition by His-p21 of DNA synthesis using purified replication proteins. *a* and *b*, Analysis of the reconstituted SV40 DNA-replication system. Purified proteins were used for reconstituted replication including replication protein (RPA), topoisomerases I and II, RFC (fraction IV), DNA polymerase-α/primase complex (pol α/primase), PCNA and pol δ. These proteins were preincubated on ice with His-p21 (*a*) or with PCNA and 19 μg ml^{-1} His-p21 (*b*), T-antigen and plasmid DNA were then added and replication assayed as for Fig. 1. *c* and *d*, Analysis of PCNA-activated DNA synthesis by purified DNA polymerase δ with oligo(dT)/poly(dA) as primer–template. DNA synthesis is expressed as picomoles TMP incorporated in a 25-μl reaction in 20 min. *c*, His-p21 was preincubated with 1 μg ml^{-1} PCNA and 1 μg ml^{-1} pol δ (filled circles), pol δ alone (circles) or control buffer (triangles) on ice for 30 min, then incubated with TTP and the homopolymer primer–template DNA at 37 °C for 20 min. *d*, PCNA and pol δ were incubated with or without 6 μg ml^{-1} His-p21 on ice before DNA synthesis.

METHODS. Reconstituted replication and DNA synthesis reactions with pol δ were carried out as described[18,21,29]. The standard reconstituted replication reaction contained 6 μg ml^{-1} pSV011 template plasmid, 80 μg ml^{-1} T-antigen, 4 μg ml^{-1} topoisomerase I, 1.8 μg ml^{-1} topoisomerase II, 50 μg ml^{-1} RPA, 8 μg ml^{-1} PCNA, 72 μg ml^{-1} RFC (fraction IV), 1 μg ml^{-1} pol α/primase complex and 5 μg ml^{-1} pol δ. These proteins were purified as before[18,29].

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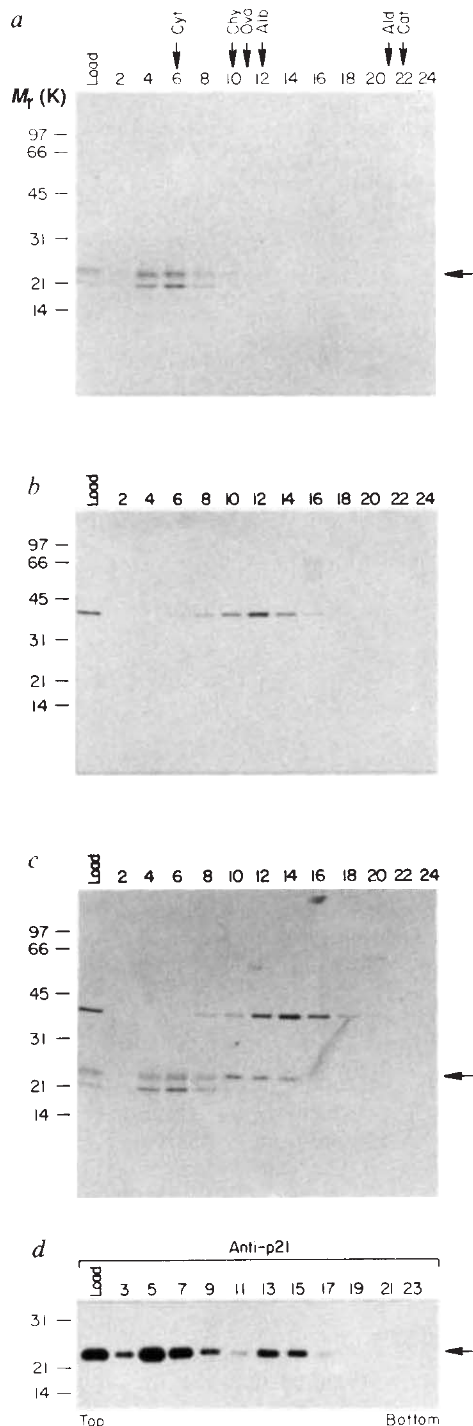

FIG. 4 Analysis of a His-p21/PCNA complex by glycerol gradient sedimentation. His-p21 (*a*), PCNA (*b*) or both proteins combined (*c*, *d*) were sedimented in parallel in 15–35% glycerol gradients containing 0.25 M NaCl. Proteins in every second fraction (out of 27 per gradient) were precipitated with trichloroacetic acid, separated by 15% SDS–PAGE, and stained with silver (top three panels). Alternative fractions containing both His-p21 and PCNA were analysed by western blotting with rabbit anti-p21 antiserum (G.H. and D.B., unpublished) (panel d). The fraction numbers are shown above each gel. Protein in the 'Load' lanes is equivalent to one fifteenth of the total protein in each gradient. Sedimentation positions of the marker proteins, cytochrome *c* (Cyt; 12.5K), chymotrypsinogen A (Chy; 25K), ovalbumin (Ova; 45K), serum albumin (Alb; 68K), aldolase (Ald; 158K) and catalase (Cat; 240K) are indicated in *a*. Migration positions of size markers in SDS–PAGE are shown on the left. Arrows in *a*, *c* and *d* indicate His-p21. The protein migrating faster than His-p21 is an unrelated *E. coli* protein that serves as an internal control; bands at ~66K in *b* and *c* are contaminants introduced in the gel electrophoresis.

METHODS. A mixture of PCNA and His-p21 (0.8 μg each, giving a molar excess of p21) or each protein alone was preincubated on ice for 2 h in a buffer consisting of 0.25 M NaCl, 25 mM Tris-HCl, pH 7.5, 5% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.01% Nonidet P-40 and protease inhibitors, before sedimentation in 5-ml 15–35% glycerol gradients in the same buffer in a Beckman SW55Ti rotor for 21.5 h at 55,000 r.p.m. at 4 °C. A duplicate gradient was run in parallel with protein markers. Immuno blotting with rabbit anti-p21 antiserum followed the manufacturer's recommended protocol (ECL, Amersham).

These results encouraged us to determine whether p21 and PCNA could form a complex in solution in the absence of CDKs or cyclins using glycerol gradient sedimentation. His-p21 sedimented at the same position as a marker protein of relative molecular mass 12,500 (M_r 12.5K) (fraction 6 in Fig. 4*a*); PCNA co-sedimented with a 68K marker protein (fraction 12 in Fig. 4*b*) (PCNA forms a torus-like homo-trimer in solution[22,23]). When PCNA and His-p21 were mixed before applying to the gradient, the PCNA peak shifted to fraction 14 and cosedimented with part of the His-p21 (Fig. 4*c*). Note that a contaminating *E. coli* protein present in this His-p21 preparation did not bind PCNA. Immunoblotting with p21 antiserum confirmed that the PCNA-associated protein was His-p21 (Fig. 4*d*). The increased sedimentation constant of PCNA when bound to His-p21 or GST-p21 (data not shown) suggests that one molecule of p21 is bound to one molecule of a trimeric PCNA.

In normal non-tumorigenic human cells, p21 exists in a quaternary complex with a cyclin, a CDK and PCNA[8,24]. Because p21 binds to PCNA directly, a complex of p21 with only PCNA may exist in normal cells. The activity of the kinase in the quaternary complex depends on the amount of p21 in the complex (H. Zhang, G.J.H. and D.B., manuscript submitted), so in normal cells p21 probably controls both CDK/cyclin and PCNA activity. Thus p21 could provide a regulatory link in normal diploid cells between DNA replication and those CDK/cyclin complexes required for the execution of S phase. It has been proposed that PCNA in *S. pombe* participates in a replication checkpoint that affects entry into mitosis[25], so an alternative role for the p21/PCNA complex or the quaternary complex at the replication fork might be in mediating this checkpoint.

The level of p21 is controlled by the tumour-suppressor protein p53 (refs 1, 4), and our observations suggest a mechanism whereby p53 could modulate and perhaps coordinate cell-cycle progression and DNA replication. In tumour cells that have either lost the p53 protein or contain an altered form of p53, p21 levels are greatly reduced or the protein is absent altogether[1,4,5]. The consequences of this could be abnormal DNA replication control or loss of coordination between DNA replication and cell-cycle progression. Both could lead to genome instability, a characteristic of tumour cells.

Following DNA damage by ultraviolet light, γ-rays or chemicals, cells respond by activating DNA repair processes that are partly controlled by p53[26-28]. Induction of p21 by p53 could be one mechanism by which the cell cycle[1,3,4,6] and DNA replication are transiently arrested. As PCNA is required for DNA-excision repair[13], we assume either that the PCNA and DNA polymerase involved in DNA repair are refractory to inhibition by p21, or that there is a damage-induced programme that allows DNA repair before resumption of replication.

In addition to its established role as a CDK inhibitor, our results demonstrate a previously unrecognized function for p21 in the control of DNA replication. This suggests that p21 may be a key governer of DNA replication, DNA repair and the cell cycle machinery. □

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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).

© 1994 Nature Publishing Group

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. Noda, A. F., Ning, Y., Venable, S., Pereira-Smith, O. M. & Smith, J. R. *Expl Cell Res.* **211,** 90–98 (1994).
8. Xiong, Y., Zhang, H. & Beach, D. *Cell* **71,** 505–514 (1992).
9. Prelich, G., Kostura, M., Marshak, D. R., Mathews, M. B. & Stillman, B. *Nature* **326,** 471–475 (1987).
10. Prelich, G. & Stillman, B. *Cell* **53,** 117–126 (1988).
11. Tsurimoto, T., Melendy, T. & Stillman, B. *Nature* **346,** 534–539 (1990).
12. Waga, S. & Stillman, B. *Nature* **369,** 207–212 (1994).
13. Shivji, M. K. K., Kenny, M. K. & Wood, R. D. *Cell* **69,** 367–374 (1992).
14. Challberg, M. D. & Kelly, T. J. *A. Rev. Biochem.* **58,** 671–717 (1989).
15. Stillman, B. *A. Rev. Cell Biol.* **5,** 197–245 (1989).
16. Melendy, T. & Stillman, B. in *Nucleic Acids and Molecular Biology* Vol. VI (eds Eckstein, F. & Lilley, D. M. J.) 129–158 (Springer, Berlin, 1992).
17. Hurwitz, J., Dean, F. B., Kwong, A. D. & Lee, S.-H. *J. biol. Chem.* **265,** 18043–18046 (1990).
18. Waga, S., Bauer, G. & Stillman, B. *J. biol. Chem.* **269,** 10923–10934 (1994).
19. Stillman, B. W. & Gluzman, Y. *Molec. cell. Biol.* **5,** 2051–2060 (1985).
20. Serrano, M., Hannon, G. J. & Beach, D. *Nature* **366,** 704–707 (1993).
21. Tan, C.-K., Castillo, C., So, A. G. & Downey, K. M. *J. Biol. Chem.* **261,** 12310–12316 (1986).
22. Bauer, G. A. & Burgers, P. M. J. *Proc. natn. Acad. Sci. U.S.A.* **85,** 7506–7510 (1988).
23. Kong, X.-P., Onrust, R., O'Donnell, M. & Kuriyan, J. *Cell* **69,** 425–437 (1992).
24. Zhang, H., Xiong, Y. & Beach, D. *Molec. Biol. Cell* **4,** 897–906 (1993).
25. Waseem, N. H., Labib, K., Nurse, P. & Lane, D. P. *EMBO J.* **11,** 5111–5120 (1992).
26. Kastan, M. *et al.* *Cell* **71,** 587–597 (1992).
27. Kuerbitz, S. J., Plunkett, B. S., Walsh, W. V. & Kastan, M. B. *Proc. natn. Acad. Sci. U.S.A.* **89,** 7491–7495 (1992).
28. Lu, X. & Lane, D. P. *Cell* **75,** 765–778 (1993).
29. Tsurmoto, T. & Stillman, B. *J. biol. Chem.* **266,** 1961–1968 (1991).

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RAD25 is a DNA helicase required for DNA repair and RNA polymerase II transcription

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THE *RAD25* gene of *Saccharomyces cerevisiae* functions in nucleotide excision repair of ultraviolet-damaged DNA and is also required for cell viability[1]. The RAD25 protein shows remarkable homology to the protein encoded by the human nucleotide-excision-repair gene *XPB* (*ERCC3*), mutations in which cause the cancer-prone disease xeroderma pigmentosum and also Cockayne's syndrome[1]. Here we purify RAD25 protein from *S. cerevisiae* and show that it contains single-stranded DNA-dependent ATPase and DNA helicase activities. Extract from the conditional lethal mutant *rad25-ts$_{24}$* exhibits a thermolabile transcriptional defect which can be corrected by the addition of RAD25 protein, indicating a direct and essential role of RAD25 in RNA polymerase II transcription. The protein encoded by the *rad25$_{799am}$* allele is defective in DNA repair but is proficient in RNA polymerase II transcription, indicating that RAD25 DNA-repair activity is separable from its transcription function. The *rad25 Arg-392* encoded product, which contains a mutation in the ATP-binding motif, is defective in RNA polymerase II transcription, suggesting that the *RAD25*-encoded DNA helicase functions in DNA duplex opening during transcription initiation.

The RAD25 (SSL2) protein was overproduced in *S. cerevisiae* (Fig. 1*a*), and purified almost to homogeneity (Fig. 1*b*). RAD25 protein in the overproducing extract (Fig. 1*a*, lane 2) and RAD25 protein immunoprecipitated from wild-type extract (Fig. 3*a*, lane 1) both show an apparent molecular size of 105K in SDS–PAGE analysis, which is consistent with the predicted size of 95.3K[1]. The identity of purified RAD25 protein was established by immunoblotting (Fig. 1*a*, lane 3).

To examine whether RAD25 protein has ATPase activity, it was incubated with [2,5,8-^{3}H]ATP in the absence of DNA and with either double-stranded (ds) or single-stranded (ss) DNA as cofactor. As shown in Table 1, RAD25 hydrolyses ATP to ADP in the presence of DNA but not in its absence, with ssDNA being much more effective than dsDNA in activating ATPase activity. The ssDNA-dependent ATPase activity of RAD25 protein requires Mg^{2+} (Table 1), is maximal at pH 7.25 (data not shown), and has $k_{cat} = 140\ min^{-1}$ at this pH. The level of ssDNA-dependent ATPase activity in the Mono-Q fractions from the last step of RAD25 purification was found to parallel closely the amount of RAD25 (Fig. 2*a*), indicating that this activity is intrinsic to RAD25 protein. RAD25 protein also hydrolyses dATP with similar efficiency and the same DNA cofactor requirement (data not shown).

TABLE 1 RAD25 protein is a ssDNA-dependent ATPase

Conditions	ATPase activity (%)
No DNA	0
Single-stranded DNA	
M13 mp18	100
ΦX174	100
ΦX174 ($-Mg^{2+}$)	0
Duplex DNA	
pBR322	5
M13mp18	7
ΦX174	8
ΦX174 ($-Mg^{2+}$)	0

Reaction mixtures (10 µl final volume) containing 20 ng RAD25 protein, 200 ng of the indicated DNA co-factor, and 0.25 mM [2,5,8-^{3}H]ATP (10^5 c.p.m. total) were assembled in 30 mM Tris-HCl, pH 7.25, containing 5 mM $MgCl_2$, 1 mM dithiothreitol and 100 µg ml^{-1} BSA and incubated for 30 min at 36 °C. The duplex DNA contained 80% supercoiled form and 20% nicked circular form. Conversion of ATP to ADP was determined by thin layer chromatography in polyethyleneimine cellulose sheets; 100% activity corresponds to the hydrolysis of 35% of the input ATP.

Many ssDNA-dependent ATPases have the ability to unwind duplex DNA[2]. To examine whether RAD25 has DNA helicase activity, we incubated it with ssM13 circular DNA to which a ^{32}P-labelled 17-nucleotide complementary fragment had been annealed. Figure 2*b* shows that RAD25 protein unwinds the partial duplex (lanes 4 and 5) in an ATP-dependent manner (compare lane 3 with lane 5). Replacing ATP with the non-hydrolysable ATP analogue ATP-γS inactivates DNA unwinding (lane 6), suggesting that the energy derived from hydrolysing the β–γ phosphodiester bond in ATP drives the helicase function. Whereas dATP (lane 7) substitutes for ATP in supporting DNA unwinding, CTP (lane 8), GTP (lane 9), UTP (lane 10) and ADP (data not shown) are ineffective. As shown in Fig. 2*c*, RAD25 protein also catalyses the ATP-dependent unwinding of another helicase substrate which contains a 41-base-pair (bp) duplex region. The helicase activity requires Mg^{2+}, has a pH 7.25 optimum, and co-elutes with RAD25 protein during chromatography in Mono-Q (data not shown).

Our previous studies with the recessive temperature-sensitive (ts) conditional lethal mutation *rad25-ts$_{24}$* have indicated a general requirement for RAD25 in RNA polymerase II transcription *in vivo*[3]. Here we determine whether RAD25 functions directly in RNA polymerase II transcription. Using immunopre-

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