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18. Plasmid EPOenv Δ5923 was constructed by use of unique restriction sites at position 5923 (Bst EII) and position 6537 (Bam HI) of the Mo-MuLV *env* gene to delete the intervening envelope sequence and allow the EPO sequence to be inserted, in frame, at the $NH_2$-terminal end of gp70. The EPO cDNA sequence coding for the mature 166–amino acid peptide hormone, without the 27–amino acid signal peptide, was used to replace the Mo-MuLV *env* sequences that had been deleted. An EPO sequence with compatible ends was created by polymerase chain reaction (PCR)–mediated mutagenesis, with 5′-GGCCTC-CCAGTGGTAACCGCCCCACCACGC-3′ as the 5′ primer and 5′-GGACACTCCTGGGATCCTGTCCC-CTGTCCT-3′ as the 3′ primer, followed by restriction digest of the PCR product with Bst EII and Bam HI, so that it could be inserted by means of the appropriate restriction sites while maintaining the proper reading frame. We also made a second construct containing EPO in the central portion of gp70 (between positions 6257 and 6761 of the *env* gene), directly overlapping a proline-rich hypervariable region. Although a few subclones expressed this construct at the protein level, as shown by protein immunoblot analysis, none expressed it on the surface of the packaging cells, as shown by FACS analysis. This lack of cell-surface expression was presumably due to retention in the endoplasmic reticulum (*12*).
19. To create packaging cell lines expressing the recombinant envelope, $1 \times 10^6$ ψ2 cells, grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 1% penicillin-streptomycin, were cotransfected with 20 μg of pEPOenv Δ5923 and 1 μg of pFR400 by means of the calcium phosphate precipitation method (Gibco). The medium was changed 16 to 24 hours later and was subsequently selected with gradually increasing concentrations of MTX (Sigma) from 0.2 to 10 μM, at which point subclones were isolated from the surviving colonies and screened by protein immunoblot. Subclones were grown to confluence in 10-cm plates, washed with phosphate-buffered saline (PBS), and lysed in 4% SDS, 10% glycerol, 10% β-mercaptoethanol, and 50 mM tris (pH 6.7) and boiled for 10 min. The samples were subjected to 8% SDS-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose (Hybond ECL, Amersham). The filters were blocked in TBST buffer [0.02% Tween-20, 150 mM NaCl, and 50 mM tris (pH 7.5)] with 5% dried milk and incubated with a primary EPO mAb (Genzyme), then washed in TBST buffer with 0.5% dried milk and incubated with a secondary horseradish peroxidase–conjugated goat antibody to mouse immunoglobulin G (IgG) (CalTag, San Francisco, CA). After being washed again, the filters were incubated in ECL chemiluminescent immunodetection reagents (Amersham) and exposed to film. The same specific 70-kD band was also observed when the filters were reblocked and reprobed with a polyclonal goat anti-*env* (Microbiological Associates, Rockville, MD) as the primary antibody, followed by horseradish peroxidase–conjugated swine antibody to goat IgG (CalTag) as the secondary antibody.
20. Polyclonal antiserum to EPO no. 8C295 (Amgen) and fluorescein-conjugated secondary antibodies (CalTag) were used for flow cytometry, carried out with a Becton Dickinson FACScan and FACS IV, operated by P. Dazin.
21. Virus-producing cell lines were created by transient transfection of amphotropic packaging cell line PA317 with pCRIP-SVlac (which contains the gene for neomycin resistance and the gene for β-Gal and is a modification of a retroviral vector plasmid provided by R. Scharfmann) by means of the calcium phosphate precipitation method. The medium was changed after 24 hours, and the virus-containing medium was filtered through a 0.2-μm filter into medium containing polybrene (8 μg/ml) and then used to infect wild-type ψ2 as well as ψEPOenv 8.1 packaging cells 48 hours after transfection. The infected packaging cells were subsequently replated at low density and selected in G418 (400 μg/ml) (Gibco), and individual G418-resistant colonies were isolated. Subcloned colonies were retested for expression of the EPO epitope by protein immunoblot and FACS analysis. Amphotropic, wild-type virus-producing cells were created by a similar strategy that used transient transfection of wild-type ψ2 cells with pCRIP-SVlac to generate virus for infection of PA317 packaging cells, followed by G418 selection.
22. NIH 3T3 cells were cotransfected with pXM EPO-R and pFR400 and selected in increasing concentrations of MTX ranging from 0.2 to 10 μM. Expression of the EPO receptor was assayed by binding of $^{125}$I-EPO peptide (Amersham); target cells were grown to confluence in 24-well plates and subsequently incubated in DMEM with 2% bovine serum albumin (BSA) and approximately $10^6$ cpm of $^{125}$I-EPO, with or without excess cold EPO, for 90 min at 37°C in a humidified incubator, washed with PBS, lysed in 1N NaOH, and counted in a gamma counter. The approximate number of EPO receptors per cell was estimated on the basis of the specific activity of the $^{125}$I-EPO and the approximate number of cells contained within the wells. HEL and K562 cells were similarly assayed for $^{125}$I-EPO binding, except that cells were incubated with $^{125}$I-EPO in suspension and were spun for 5 min at 1000 rpm in a Beckman desktop centrifuge to pellet the cells after each wash.
23. The ψ2 wild-type control virus showed titers of $3.1 \times 10^4$ per milliliter and $2.8 \times 10^4$ per milliliter on NIH 3T3 wild-type and NIH 3T3 + EPO receptor target cells, respectively. The ψ2 wild-type virus titers were not affected by the presence or absence of EPO. The EPO-*env* virus showed a titer of $1.8 \times 10^4$ per milliliter on NIH 3T3 wild-type cells and $1.2 \times 10^5$ per milliliter on NIH 3T3 + EPO receptor cells without EPO competition. With EPO competition, the EPO-*env* virus titers on NIH 3T3 wild-type cells were not significantly affected; however, the titers on NIH 3T3 + EPO receptor cells were drastically reduced, to $6.2 \times 10^4$ per milliliter with the addition of 5 μg of EPO per milliliter and $5.5 \times 10^4$ per milliliter with the addition of 50 μg of EPO per milliliter. Two variables were then taken into account in interpreting these results and determining the efficiency of infection. First, differences in initial plating density and growth rates of the two target cell lines were corrected for by comparison of the number of G418-resistant NIH 3T3 + EPO receptor colonies with the number of G418-resistant NIH 3T3 wild-type colonies after infection by ψ2 wild-type virus. Second, differences in the overall titers of the wild-type and EPO-*env* viruses were corrected for by comparison of the number of G418-resistant NIH 3T3 wild-type colonies after infection with ψ2 virus with that of NIH 3T3 wild-type colonies after infection with EPO-*env* virus. Thus, the relative infection efficiency of EPO-*env* virus on NIH 3T3 + EPO receptor cells as compared with that of ψ2 wild-type virus was derived by the following formula: relative infection efficiency = (number of G418-resistant NIH 3T3 + EPO receptor colonies per number of G418-resistant NIH 3T3 wild-type colonies) after EPO-*env* virus infection ÷ (number of G418-resistant NIH 3T3 + EPO receptor colonies per number of G418-resistant NIH 3T3 wild-type colonies) after ψ2 wild-type virus infection. The relative infection efficiency thus derived, expressed as the mean ± SE, was 6.1 ± 1.2 ($n$ = 6).
24. We thank J. Chang for help in retrovirus work and T. Reudelhuber, D. Gardner, K. Yamamoto, and J. A. T. Young for stimulating discussions and helpful advice. Plasmids containing Mo-MuLV genes were generously provided by A. Bank and D. Markowitz. A plasmid containing EPO cDNA sequences was generously provided by K. Shannon. Plasmids containing the murine and human EPO receptor cDNAs were generously provided by A. D'Andrea. Polyclonal antiserum to EPO no. 8C295 was generously provided by J. Egrie. Supported in part by NIH grants HL 20985 (to the Northern California Comprehensive Sickle Cell Center) and AM16666 (to Y.W.K., who is an investigator at the Howard Hughes Medical Institute).

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# Interaction of the p53-Regulated Protein Gadd45 with Proliferating Cell Nuclear Antigen

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*GADD45* is a ubiquitously expressed mammalian gene that is induced by DNA damage and certain other stresses. Like another p53-regulated gene, $p21^{WAF1/CIP1}$, whose product binds to cyclin-dependent kinases (Cdk's) and proliferating cell nuclear antigen (PCNA), *GADD45* has been associated with growth suppression. Gadd45 was found to bind to PCNA, a normal component of Cdk complexes and a protein involved in DNA replication and repair. Gadd45 stimulated DNA excision repair in vitro and inhibited entry of cells into S phase. These results establish *GADD45* as a link between the p53-dependent cell cycle checkpoint and DNA repair.

An important cellular response to DNA damage is the arrest of cell cycle progression at $G_1$ and $G_2$ checkpoints, which presumably allows time for DNA repair before entry into S and M phase, respectively (*1*). The p53 tumor suppressor is required for one such $G_1$ checkpoint in mammalian cells (*2*, *3*). After genotoxic stress, p53 functions as a transcription factor and transactivates effector genes such as *GADD45* and $p21^{WAF1/CIP1}$, although both of these genes can be induced by other pathways (*3*, *4*). $p21^{Waf1/Cip1}$ inhibits the kinase activity of multiple Cdk complexes, which may be one mechanism by which it suppresses cellular growth (*5*, *6*), and it inhibits the ability of PCNA to activate

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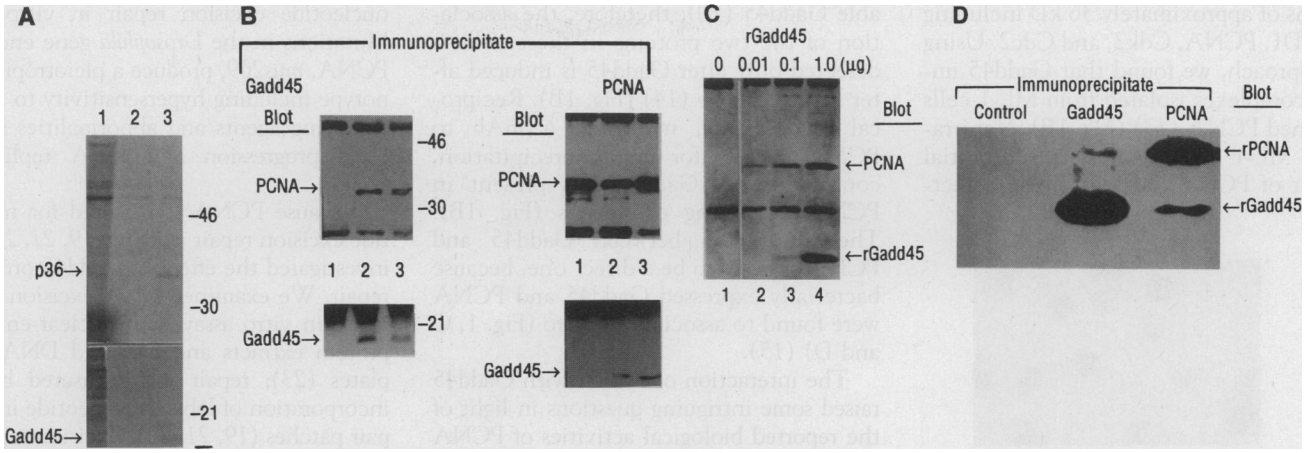


**Fig. 1.** (**A**) Coimmunoprecipitation of Gadd45 and p36 from $^{35}$S-methionine–labeled cells with Gadd45 mAb 30T14. Lane 1, total cell lysate before immunoprecipitation; lane 2, immunoprecipitation with control mAb; and lane 3, immunoprecipitation with mAb 30T14 (*10*). ML-1 cells were γ-irradiated with 20 Gy and incubated with $^{35}$S-methionine (300 μCi/ml) for 4 hours. (**B**) Immunoprecipitation and immunoblot analysis of the Gadd45-PCNA interaction (*13*). ML-1 cells were treated as in (A). Lanes 1, nonirradiated cells; lanes 2, cells harvested 3 hours after irradiation with 20 Gy of ionizing radiation from a $^{137}$Cs source; and lanes 3, cells harvested 3 hours after irradiation with 8 J m$^{-2}$, using a 254-nm UV source. The lower half of the blot was probed with Gadd45 hybridoma supernatant 4T (*10*); the upper half of the blot was probed with PCNA mAb PC-10. Detection was with goat antibody to mouse immunoglobulin G coupled to horseradish peroxidase in combination with enhanced chemiluminescence (Amersham). (**C**) In vitro incubation of recombinant Gadd45 (rGadd45) with extracts from nonirradiated ML-1 cells, followed by immunoprecipitation with mAb 30T14, results in the coimmunoprecipitation of cellular PCNA (*15*). (**D**) Association of rGadd45 and recombinant PCNA (rPCNA) in vitro shown by coimmunoprecipitation (*15*) with detection by immunoblotting as above.

DNA polymerase δ for viral DNA replication in vitro (*7*). The mechanism of action of *GADD45* is unknown. In addition to growth suppression, another potentially important cellular response to genotoxic stress is the enhancement or induction of DNA repair which, while most pronounced in prokaryotes (*8*), has also been detected in mammalian cells (*9*). Here, we investigate the function of the Gadd45 protein, with particular emphasis on its possible role in growth control and DNA repair.

We used monoclonal antibodies (mAbs) to Gadd45 to investigate interactions of Gadd45 with other nuclear proteins (*10*). Analysis of Gadd45 immune complexes prepared from $^{35}$S-methionine–labeled ML-1 cells [a human myeloid leukemia line containing wild-type p53 (*3*)], after ionizing radiation treatment, revealed two proteins that migrated in SDS-polyacrylamide gels at approximately 19 and 36 kD (*11*) (Fig. 1A). The 19-kD protein migrated at the position expected for Gadd45 (*10*). To identify the other protein, we sequentially immunoblotted Gadd45 immune complexes (*12*) with antibodies recognizing known

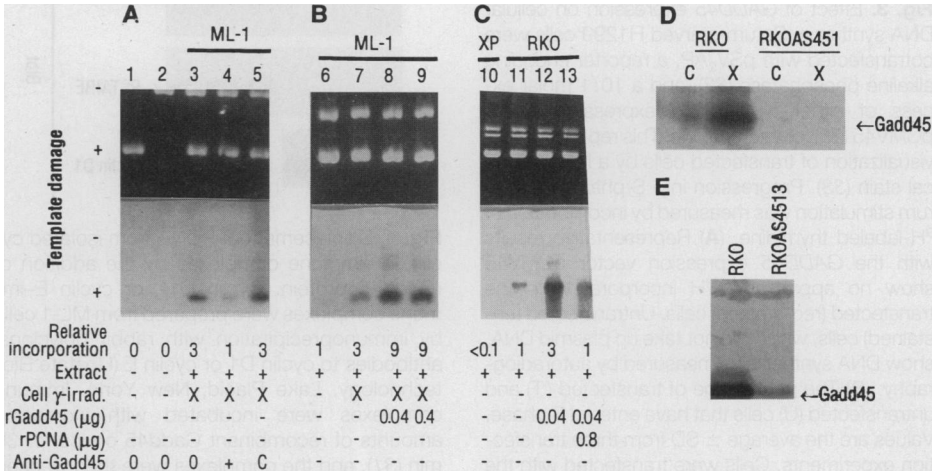


**Fig. 2.** Effect of Gadd45 on DNA excision repair in vitro (*23*). Excision repair assays were carried out as in (*22*). Damaged plasmid DNA (+) or undamaged plasmid DNA of a different size (−) were incubated with nuclear-enriched extracts prepared from nonirradiated or γ-irradiated cells. (A to C) Shown is the ethidium bromide staining of linear plasmid DNA after electrophoresis (upper photos), and the extent of DNA repair as measured by the incorporation of labeled nucleotide during repair synthesis (lower photos); appreciable repair synthesis was detected only in DNA samples containing damage. ML-1 cells were used in (A) and (B); RKO cells were used in (C), with the exception of lane 10, in which excision repair–defective XP cells of complementation group A (XP12BE cells) served as a negative control (*24*). Relative incorporation was determined by densitometry, with the basal level of incorporation for each repair-competent cell line (ML-1 or RKO) designated as 1.0. Thus, values greater than 1.0 indicate increased repair activity, and values less than 1.0 indicate reduced repair. (**A**) Lanes 3 to 5 contain extracts from γ-irradiated ML-1 cells; lanes 1 and 2 contain controls without cell extracts. In lane 4, Gadd45 was immunodepleted from the cell lysate before the repair incubation; in lane 5, an unrelated antibody control was used for immunodepletion. Plasmid DNA was damaged by exposure to 254-nm UV irradiation at 1000 J m$^{-2}$ and then treated with *E. coli Nth* protein (*22*) to remove extraneous photoproducts. The remaining supercoiled plasmid was isolated and used in the assay. (**B**) Bacterially expressed recombinant Gadd45 protein (rGadd45) was added to the reactions in lanes 8 and 9. Plasmid DNA was damaged by treatment with 0.1 mM *N*-acetoxy-2-acetylaminofluorene (AAF) (*22*). γ-Irradiation of ML-1 cells (indicated by X) was as in Fig. 1. (**C**) Effect of Gadd45 addition on repair synthesis by extracts from nonirradiated RKO cells (lanes 11 to 13) or from XP controls (lane 10). Plasmid DNA was damaged by treatment with AAF. (**D**) Reduction of Gadd45 expression by antisense RNA in RKOAS451 cells. Cells were either nonirradiated (C) or were treated with ionizing radiation (X); detection was by immunoprecipitation and immunoblotting as in Fig. 1. (**E**) Reduction of Gadd45 expression by antisense RNA in nonirradiated RKOAS4513 cells. Detection was by immunoblotting of nuclear extract proteins (200 μg). Exposure time in (E) was extended to show Gadd45 levels in nonirradiated cells; an additional band of approximately 32 kD is visible at this exposure, which was not altered by *GADD45* antisense expression.

proteins of approximately 36 kD including cyclin D1, PCNA, Cdk2, and Cdc2. Using this approach, we found that Gadd45 immune complexes isolated from ML-1 cells contained PCNA (*13*) (Fig. 1B). Nonirradiated ML-1 cells express a substantial amount of PCNA, but very little detectable Gadd45 (*10*); therefore, the association of the two proteins in these cells is detected only after Gadd45 is induced after DNA damage (*14*) (Fig. 1B). Reciprocal experiments, in which a mAb to PCNA was used for immunoprecipitation, confirmed that Gadd45 was present in PCNA-containing complexes (Fig. 1B). The interaction between Gadd45 and PCNA appears to be a direct one, because bacterially expressed Gadd45 and PCNA were found to associate in vitro (Fig. 1, C and D) (*15*).

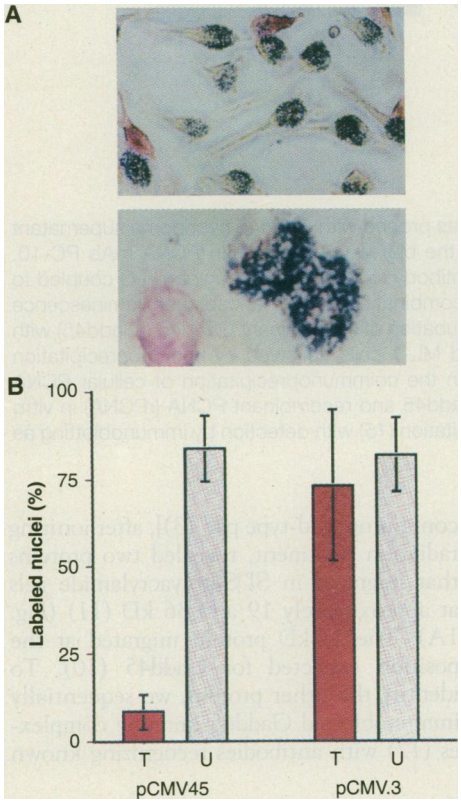


**Fig. 3.** Effect of *GADD45* expression on cellular DNA synthesis. Serum-starved H1299 cells were cotransfected with $pSV_2AP$, a reporter encoding alkaline phosphatase (*33*), and a 10:1 molar excess of either a *GADD45* expression vector pCMV45 (*29*), or vector alone. This reporter allows visualization of transfected cells by a histochemical stain (*33*). Progression into S phase after serum stimulation was measured by incorporation of $^3$H-labeled thymidine. (**A**) Representative results with the *GADD45* expression vector pCMV45 show no appreciable $^3$H incorporation in the transfected (red-stained) cells. Untransfected (unstained) cells, which did not take up plasmid DNA, show DNA synthesis as measured by autoradiography. (**B**) The percentage of transfected (T) and untransfected (U) cells that have entered S phase. Values are the average ± SD from three transfection experiments. Cells were transfected with the plasmid mixtures (2 μg), using Lipofectamine reagent (BRL Gibco) in serum-free Ham's F-12 medium. Cells were incubated in the absence of serum for 72 hours to arrest them in a $G_0$-$G_1$ state, and then given medium containing 10% fetal bovine serum and [$^3$H]thymidine (1 μCi/ml). H1299 cells, which have a short doubling time, were incubated for 9 hours, allowing time for quiescent cells to enter S phase. At that time, the cells were fixed with methanol, stained for alkaline phosphatase reporter, and then processed for autoradiography.

The interaction of PCNA with Gadd45 raised some intriguing questions in light of the reported biological activities of PCNA in DNA replication and repair. PCNA is a component of multiple cyclin-Cdk complexes in normal cells (*16*) and has been associated with viral and cellular replication origins (*17*). After DNA damage, an apparent change in the nuclear distribution of PCNA is evident by immunostaining, which may indicate a redistribution of PCNA from sites of DNA replication to sites of DNA damage (*18*). PCNA is an auxiliary factor for repair-associated DNA polymerases δ and ε and is required for nucleotide excision repair in vitro (*19*). Mutations in the *Drosophila* gene encoding PCNA, *mus209*, produce a pleiotropic phenotype including hypersensitivity to DNA-damaging agents and abnormalities in cell cycle progression and DNA replication (*20*).

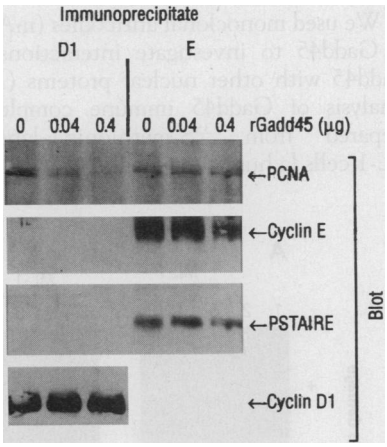


**Fig. 4.** Displacement of PCNA from isolated cyclin D1–immune complexes by the addition of rGadd45 protein. Cyclin D1– or cyclin E–immune complexes were prepared from ML-1 cells by immunoprecipitation with rabbit polyclonal antibodies to cyclin D1 or cyclin E (Upstate Biotechnology, Lake Placid, New York). Immune complexes were incubated with increasing amounts of recombinant Gadd45 on ice for 30 min (*31*), and the complexes were then washed once with 1 ml of lysis buffer before SDS–polyacrylamide gel electrophoresis and transfer to nitrocellulose. Individual components of the immune complexes were revealed by immunoblotting. Cyclin D1 was detected with the same antibody used for immunoprecipitation; cyclin E was detected with a mouse mAb (Pharmingen, San Diego, California); PCNA was detected with PC-10 (Santa Cruz Biotech, Santa Cruz, California); and Cdc2-like proteins (such as Cdk2) were detected with a mAb to the PSTAIRE-containing sequence (Upstate Biotechnology).

Because PCNA is required for nucleotide excision repair in vitro (*19*, *21*, *22*), we investigated the effect of Gadd45 on DNA repair. We examined DNA excision repair in an in vitro assay with nuclear-enriched protein extracts and damaged DNA templates (*23*); repair was measured by the incorporation of labeled nucleotide into repair patches (*19*, *21*, *22*). Excision repair in vitro was reduced approximately threefold by immunodepletion of nuclear-enriched extracts with a mAb to Gadd45 (Fig. 2A), and repair was enhanced three- to fivefold by the addition of recombinant Gadd45 protein to the assay (Fig. 2, B and C). The addition of recombinant Gadd45 stimulated excision repair irrespective of whether extracts were prepared from irradiated cells or from nonirradiated cells. Interestingly, extracts from nonirradiated ML-1 cells showed appreciably less repair activity than did extracts from nonirradiated RKO cells, which contain higher levels of Gadd45 (*10*). Extracts from repair-deficient cells (*24*), included as a control, were found to have very low activity (Fig. 2C). When extracts from irradiated ML-1 cells were used, repair activity was appreciably increased, reflecting an inducible repair-like response, and the levels approached that of the nonirradiated RKO cells. Whereas the addition of PCNA alone had no effect, the simultaneous addition of excess recombinant PCNA (*25*) blocked the ability of Gadd45 to stimulate repair (Fig. 2C), probably because excess recombinant PCNA blocked Gadd45 interaction with the PCNA-containing repair complexes. It is not clear whether Gadd45 has a direct or indirect role in repair; conceivably, it could facilitate the formation of PCNA-containing repair complexes or it may itself associate with the DNA polymerase δ repair complex.

In vivo evidence for a role of Gadd45 in repair of ultraviolet (UV)-type lesions was obtained through the use of two RKO cell lines in which Gadd45 induction and basal expression were blocked by high-level constitutive expression of *GADD45* antisense RNA (Fig. 2, D and E) (*26*). Both antisense cell lines always showed reduced survival as compared to the parent line after 20 $Jm^{-2}$ of UV radiation in five different experiments that were highly significant ($P$ = 0.0047 for pooled data) (*27*). Presumably, the reduced Gadd45 levels in the antisense cell lines adversely affected some aspect of repair-mediated survival at this dose of ra-

diation, which produces relatively high levels of photoproducts that are subsequently repaired.

Activation of p53 strongly inhibits cell growth, an effect that may be attributable to downstream effectors such as $p21^{Waf1/Cip1}$ (*5*, *28*). Other downstream effectors, such as Gadd45, may also act in the pathway by which p53 induces growth arrest, because *GADD45* overexpression suppresses cell growth in stably transfected cells (*29*). With this approach, the magnitude of the growth suppression by Gadd45 was comparable to that of $p21^{Waf1/Cip1}$, but appreciably less than that of p53 in the same expression vector (*6*). We assayed the effect of *GADD45* overexpression on cellular DNA synthesis by transient transfection assay (Fig. 3), because the short-term nature of this approach allows cell cycle effects to be assayed before any compensatory alterations that may occur in longer term assays; this approach also determines if overexpression can block progression from $G_0$ to S phase. Cells that were cotransfected with *GADD45* and an alkaline phosphatase reporter construct showed decreased [$^3$H]thymidine uptake upon serum stimulation as compared to control cells (Fig. 3). Thus, under these conditions Gadd45 suppressed entry into S phase, possibly by interacting with components of the cell cycle machinery that normally govern $G_1$-S phase transition.

The mechanism by which Gadd45 inhibits cell growth is not clear. In contrast to $p21^{Waf1/Cip1}$, which associates with Cdk complexes and inhibits kinase activity (*5*), Gadd45 was not detectable in Cdc2, Cdk2, or cyclins D, E, or A immune complexes, and it did not inhibit cyclin E-Cdk2–dependent activity when added to in vitro kinase assays (*30*). Therefore, the growth-inhibitory effects of Gadd45 are apparently not due to association with or inhibition of Cdk's. Perhaps significantly, $p21^{Waf1/Cip1}$ has been shown to inhibit DNA replication in vitro by a second mechanism that is independent of its Cdk inhibitory activity, but dependent on PCNA interaction (*7*). Gadd45 growth suppression may similarly be mediated by PCNA, although growth suppression in vivo may be complex.

Gadd45 might bind to PCNA molecules that are not within Cdk complexes; alternatively, PCNA might dissociate from Cdk complexes upon association with Gadd45. We found that PCNA was displaced from cyclin D1 immune complexes, but not from cyclin E immune complexes, by the addition of recombinant Gadd45 (Fig. 4). These results suggest that Gadd45 might affect PCNA interaction with some Cdk complexes (*31*), although it must be noted that the recombinant Gadd45 added to in vitro complexes exceeded cellular concentrations.

The finding that Gadd45 can associate with PCNA contributes to our understanding of how this p53-regulated protein might affect cell growth and DNA repair. DNA damage leads to increased levels of Gadd45 and $p21^{Waf1/Cip1}$ proteins (*6*, *10*). Growth suppression by $p21^{Waf1/Cip1}$ is due at least in part to its inhibition of Cdk activity (*5*). Although Gadd45 does not appear to bind stably to Cdk complexes, its interaction with PCNA may have some conformational effect on PCNA in these complexes or may actually displace it from certain complexes. Gadd45 may interact with free PCNA or may recruit PCNA from other compartmentalized pools. Such interaction may facilitate the involvement of PCNA in DNA repair. The sequestration of PCNA by Gadd45 may also result in its depletion from replication complexes, which could account for growth inhibition. PCNA appears to exist in several different forms (*32*); given that there is much more PCNA in the cell than Gadd45, it seems likely that Gadd45 interacts with a specific form of PCNA. The biological activities associated with Gadd45—enhancement of DNA repair and inhibition of cellular DNA synthesis—may be directly attributable to its interaction with PCNA. Our results indicate that the p53-mediated DNA-damage response includes an inducible DNA repair component.

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11. Neither protein was observed when the antibody was preincubated with excess recombinant Gadd45 protein, indicating specificity of this mAb for Gadd45.
12. Each lane contains immunoprecipitates from 5 mg of soluble protein prepared from $5 \times 10^7$ cells in 50 mM tris-HCl (pH 8.0), 150 mM NaCl, 0.02% $NaN_3$, phenylmethylsulfonyl fluoride (100 μg/ml), aprotinin (1 μg/ml), 1% NP-40. Immune complexes were collected by overnight agitation with protein A–agarose (Oncogene Science, Uniondale, NY) at 4°C with 1 μg of Gadd45 mAb (30T14) or PCNA mAb (PC-10) (Santa Cruz Biotech, Santa Cruz, CA). Immunoprecipitated proteins were subjected to electrophoresis on 12% SDS-polyacrylamide gels before immunoblot analysis.
13. The 36-kD protein was not observed in control experiments where extracts were first immunodepleted of PCNA. Association between PCNA and Gadd45 was demonstrated in several human cell lines (ML-1 myeloid leukemia, RKO colon carcinoma, CEM lymphoblastoid, H1299 lung carcinoma, WI-L2-NS lymphoblastoid, and CA46 Burkitt lymphoma) and in normal human fibroblasts (AG1522); in all cells, the amount of PCNA detected in Gadd45 immune complexes was proportional to the level of Gadd45. This association could be seen in nonirradiated cells, such as RKO or WI-L2-NS cells, which express higher levels of Gadd45 than do ML-1 cells. The association of Gadd45 with PCNA was stable in 1% NP-40, but was disrupted by the addition of ionic detergents.
14. Increased PCNA binding to Gadd45 did not require DNA damage, because PCNA binding to Gadd45 was observed in RKO cells that were stably transfected with a *GADD45* expression vector driven by a sheep metallothionein gene promoter, after induction of *GADD45* expression by treatment with 90 μM $ZnSO_4$ for 24 hours (C.-Y. Chen and M. B. Kastan, unpublished results).
15. Lysates from nonirradiated ML-1 cells, which contain little or no Gadd45, were prepared by sonication in phosphate-buffered saline (PBS) containing protease inhibitors. Increasing amounts of recombinant Gadd45 were added to 5-mg samples of soluble proteins and allowed to incubate on ice for 1 hour. Samples were then diluted 50-fold in 1% NP-40 lysis buffer and subjected to immunoprecipitation with mAb 30T14. For in vitro binding studies, 500 ng each of recombinant Gadd45 and recombinant PCNA were mixed in PBS containing 2% glycerol, 0.2 mM EDTA, 0.01% NP-40, and 0.1 mM dithiothreitol (DTT). After incubation on ice for 1 hour, the mixture was diluted in 1.5 ml of immunoprecipitation buffer containing 1% NP-40 and was divided into three equal portions. Immunoprecipitation of Gadd45 and PCNA was with 30T14 and PC-10, respectively. Immunoprecipitation with protein A–Sepharose alone with no antibody served as a negative control.
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23. Mixtures of damaged DNA (Bluescript SK− plasmid) and undamaged DNA (an unrelated plasmid of 5.5 kb) (1 μg each) were incubated in a 50-μl reaction containing nuclear extract (50 μg) and 45 mM Hepes (pH 8.0), 70 mM KCl, 7.4 mM $MgCl_2$, 1 mM DTT, 0.4 mM EDTA, 20 μM each of deoxyguanosine triphosphate, deoxyadenosine triphosphate (dATP), and thymidine triphosphate, 8 μM deoxycytidine triphosphate, 10 μCi of [α-$^{32}$P]dATP, 2 mM ATP, 40 mM phosphocreatine, 2.5 μg of creatine phosphokinase, 3.4% glycerol, and 18 μg of bovine serum albumin. Excision repair reactions were incubated for 3 hours at 30°C, extracted sequentially with phenol, phenol-chloroform, and chloroform alone, ethanol precipitated, and linearized with Hind III. Recombinant Gadd45 was prepared in *Escherichia coli* as a histidine-tagged fusion. Purification on a nickel column and dialysis in PBS resulted in a preparation of approximately 95% purity. The histidine tag was cleaved by thrombin and the Gadd45 was repurified.
24. Extracts from xeroderma pigmentosum (XP) cell lines, which are defective in excision repair, were included as controls. When used singly, extracts representing XP complementation groups A (XP12BE cells), B (XP11BE), or C (XP1BE) had little activity in the in vitro assay; when combined, however, the extracts showed activity similar to that of repair-competent RKO cells.
25. Addition of PCNA alone had no effect on repair synthesis in vitro, probably because cellular PCNA was already in excess. It is also conceivable that recombinant PCNA was inactive in repair complex assembly, but was active as a competitor of Gadd45.

26. Two independent clones, RKOAS451 and RKOAS4513, were isolated after transfection with pCMVas45, which expresses *GADD45* complementary DNA in the antisense orientation (*29*), in combination with a pSV2neo selectable marker. Clones were screened for high-level expression of the antisense RNA by ribonuclease protection assay. Gadd45 expression was markedly reduced in these cell lines as determined by immunoblotting. The antisense cell lines and the parent RKO cells were exposed to 254-nm UV irradiation at either 5 or 20 $Jm^{-2}$ and then grown for 7 days, at which time surviving colonies were scored after staining with crystal violet. Survival frequencies were determined by comparison with multiple dilutions of cells of each respective cell line, which were not irradiated but were otherwise identically maintained. Control cell lines consisted of the parent RKO cells, a clonal derivative of RKO, and RKO cells stably transfected with pCMV.3 vector lacking an insert. Growth rates and plating efficiencies of these different RKO lines were equivalent without irradiation. Survival frequencies were similar for each control cell line and were consistently greater than the two antisense *GADD45* cell lines at the higher UV dose, whereas survival was not appreciably different at the lower, less toxic, dose.
27. The average of the pooled absolute survival for all experiments was 0.394% for the parent line and 0.076% (reduced 5.2-fold relative to parent line) for RKOAS451 and 0.080% (reduced 4.9-fold) for RKOAS4513; absolute survival represents colony yield adjusted for plating efficiency, which was >50%. The mean and median values of cell survival relative to the parent line (determined for each experiment and then pooled) for RKOAS451 were 0.26 and 0.32 (with two-tail $P = 0.016$ by exact Wilcoxon rank-sum test for the null hypothesis of equal median relative survivals). The corresponding values for RKOAS4513 were 0.34 and 0.43 ($P = 0.032$). If the data for the two experimental lines were pooled, the values for survival relative to control were 0.30 and 0.35 ($P = 0.0047$).
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30. Increasing amounts of bacterially expressed Gadd45, ranging from 0 to 3.6 μg, were added to cyclin E–Cdk complexes, which were then tested in in vitro kinase assays with histone H1 as substrate.
31. Gadd45 that had been boiled before addition did not displace PCNA, nor did Mdm2 that was prepared in the same way.
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34. We thank B. Stillman for recombinant PCNA, K. E. Kilpatrick for her valuable contributions in the development of the mAbs, R. Cunningham for *E. coli Nth* protein, J. N. Weinstein and T. Myers for statistical analysis, A. J. Levine for the histidine-tagged *MDM2* expression plasmid, and J. Robbins for the XP cell lines. Supported in part by grants to M.B.K. from NIH (ES05777) and the Council for Tobacco Research (3187). M.B.K. is the Steven Birnbaum Scholar of the Leukemia Society of America.

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# Clonal Divergence in *Escherichia coli* as a Result of Recombination, Not Mutation

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**Nucleotide sequence analysis was performed on 12 natural isolates of *Escherichia coli* in four loci located in close proximity on the chromosome. A comparison of gene genealogies indicated that three recombination events have occurred in a subset of the strains (ECOR group A) in the time since their divergence from a common ancestor, while during the same time, no mutational divergence has occurred. The common ancestor of this subset existed no more than 2400 years ago, and recombination was shown to occur at a rate of $5.0 \times 10^{-9}$ changes per nucleotide per generation—50-fold higher than the mutation rate. Thus, recombination has been the dominant force driving the clonal divergence of the ECOR group A strains and must be considered a significant factor in structuring *E. coli* populations.**

Public policy and health decisions concerning the lateral transfer both of recombinant genes from genetically engineered microorganisms and of virulence genes from pathogens often depend on assumptions about recombination. The frequency and significance of recombination in microorganisms, however, is currently the subject of intense debate. In the *E. coli* model system, there is controversy over the extent of clonality (*1*, *2*). Clonality can be thought of as the vertical, asexual transmission of genetic material from parent to offspring, where all evolutionary change through time is strictly the result of the mutational process. In contrast, nonclonal or sexual evolution occurs by the horizontal transfer of genetic material between unrelated individuals through the recombinational process. From the perspective of the population, there is not a strict dichotomy between these two evolutionary modes. A group of strains that share a recent common ancestor and are diverging by means of the mutational process can be considered a clone. Over time, genetic material from individuals that do not share the same most recent common ancestor will be introduced into the individual members of a clone by recombination. As this occurs, the clonal background of these strains will become progressively more obscured by foreign DNA, and the clonal nature of this group will begin to deteriorate (*3*, *4*). The prevailing view of *E. coli* is that it is a primarily clonal organism. Recombination is believed to occur at such a low frequency, relative to mutation, that it is considered evolutionarily insignificant (*2*).

A misconception about the effect of recombination on the evolutionary divergence of microorganisms has further confused the issue of clonality. Recombination has often been considered incapable of breaking down clonal structure because its effect on variation is usually considered in the context of a panmictic species. Recombination is therefore considered a homogenizing force. But, recombination can also be diversifying. In a structured population, recombination within a clone will result in genetic homogenization, but recombination between individuals of different clones will cause genetic diversification within the clone. This study is concerned with the diversification of a clone when there is transfer between clones. Under these conditions, recombination can be thought of as a mechanism, complementing mutation, that generates variability within clones and drives clonal divergence.

The relative contribution of recombination and mutation to clonal divergence has been difficult to assess because of problems associated with obtaining an accurate estimate of the rate of recombination. Uncertainty exists because of the obscuring of older exchange events by more recent events and because the methods used to estimate recombination rely heavily on assumptions based on a particular population structure, even though the population structure of microorganisms is poorly understood (*5*, *6*). Milkman and Stoltzfus (*3*) and Milkman and Bridges (*4*) made the first estimates of the recombination rate in *E. coli* from nucleotide sequence data. Their calculations used estimates of the average clonal segment length (the length of a segment of DNA that recently shared ancestry with the equivalent segment from another individual) and the number of generations since their most recent common ancestor. They estimated the rate of recombination in *E. coli* to be $5 \times 10^{-12}$ transfers per nucleotide per generation (*4*). This approach determines the probability that two adjacent nucleotides originated in different lineages and were brought together by a recombination event (a parameter similar to that used in transmission genetics). Thus, the recombination rate is expressed in different units than the mutation rate. If the recombination rate is to be comparable to the mutation rate, then both rates must be expressed in terms of the rate of nucleotide change per generation (with the understanding that a single recombination event will change multiple nucleotides in batch).

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