the stimulus velocity was constant, small variations in the edge velocity may have been perceived by the animal because of the curvature of the eye.

18. The value of α, when measured in conditions of translating and looming stimuli, ranged between 2.9 and 15 rad^{-1} for angular velocities between 314 and 625 degrees s^{-1} in different animals. Single exponential fits were consistently better than linear fits; determination coefficients (r^2) are 0.962 and 0.935 for exponential fits for Figs. 2E (625 degrees s^{-1}) and 2F (−625 degrees s^{-1}) versus 0.850 and 0.835 for linear fits, respectively.
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20. The exact value of C is unimportant; it is not the absolute firing rate that matters, but the fact that the firing rate peaks and then decreases.
21. The function $f(t)$ will peak when its time derivative is 0. From Eq. 1, $df/dt = 0$ if $\dot{\theta}(t_{peak} - \delta) = \alpha\dot{\theta}^2(t_{peak} - \delta)$. From simple geometrical considerations (Fig. 1A), $\dot{\theta}(t) = (-S_{obj.} v)/(d^2 + S^2_{obj.})$ and $\ddot{\theta}(t) = (2S_{obj.} v^2 d)/(d^2 + S^2_{obj.})^2$. Equation 2, where $t_{coll.}$ is the delay before collision at the start of the movement and t_{peak} is the time when DCMD activity peaks, follows from these three equations.
22. To verify that θ represents the subtended angle of approaching objects and not the angle that separates their edges from the focus of expansion [point of null velocity during approach or recession (*27*)], we presented a composite stimulus comprising four squares around the focus of expansion. Each square was one-sixth the size of the ensemble outline. In such conditions, we found that the best fits to the data were obtained when θ represented the angular extent of each object (75% of the variance was explained by model, where C was the only free variable) and not the angle between it and the focus of expansion (14.3% of the variance was explained by model, under the same conditions).
23. It can be shown from Eq. 2 (*21*) and by using trigonometry that the angle θ_{peak} at which DCMD reaches its peak firing is related to the value of α by the following relation:

$$\text{cotg}\,(\theta_{peak}) = \frac{\alpha}{2} - \frac{|v|\,\delta}{S_{obj.}} \quad (3)$$

This relation indicates, for example, that if δ or the approach velocity v (or both) are small, the angle θ_{peak} at which the peak firing of LGMD and DCMD is attained should be constant for a wide range of object sizes. Thus, LGMD and DCMD can be considered as preferring a particular and fixed angular size (θ_{peak} = cotg^{-1} ½). It follows from Eq. 3 that, when δ is not negligible (for example, δ = 40 ms), the peak firing will not occur for a fixed value of θ. When v is constant, for example, the peak firing will occur earlier if the object is larger. When the object size is held constant, the peak firing will occur later if v is increased.

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28. Note the good agreement between data and model in all size and velocity conditions. Better fits could be obtained if α and δ were unconstrained.
29. We gratefully acknowledge C. Koch (NSF Center for Neuromorphic Systems Engineering) for use of the Hewlett-Packard workstation and D. van Essen for use of the photocell. We thank A. Braun for computer assistance and E. Schuman, C. Koch, and an anonymous referee for comments on the manuscript. Supported by NSF grant IBN-9412426 (F.G.) and an Office of Naval Research grant and NSF–Presidential Faculty Fellow Award to G.L.

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TECHNICAL COMMENTS

Does the p53 Up-Regulated Gadd45 Protein Have a Role in Excision Repair?

Martin L. Smith *et al.* (*1*) report stimulation of excision repair in DNA by Gadd45 protein. They used the repair synthesis assay that measures the preferential incorporation of nucleotides into damaged DNA compared to undamaged DNA. As DNA excision repair involves two basic steps, excision and resynthesis, we wished to know whether the increased repair synthesis observed with Gadd45 resulted from increased excision or was a secondary effect of stimulation of the repair polymerase (or polymerases) without actually increasing the amount of adducts removed. We investigated the effects of Gadd45 by the use of the excision assay that measures the release of the damaged nucleotide in the form of an oligonucleotide (*2*).

We measured the effect of Gadd45 at various concentrations by the excision assay with HeLa cell-free extracts (Fig. 1). We did not observe any stimulation or inhibition within the concentration range used. As Smith *et al.* (*1*) report stimulation of Gadd45 in the range of 40 to 400 ng per assay, we conducted a kinetic experiment using 340 ng of the Gadd45 protein in our standard excision reaction. Gadd45 had no effect on the kinetics of excision repair (Fig. 2).

To eliminate the possibility of experimental artifacts resulting from nonrepair proteins in cell-free extracts that can bind to Gadd45 and interfere with its repair stimulatory effect, we also tested the effect of Gadd45 protein on repair, with the use of a defined excision nuclease system reconstituted from highly purified repair proteins (*2*). We saw no effect on excision repair in this system with the concentration of Gadd45 tested. We considered that the stimulatory effect could be unique to the cell lines used by Smith *et al.* (*1*). Therefore, we performed the excision assay with the ML-1 cell line used by Smith *et al.* (*1*). The cell-free extract from this cell line gave a weaker excision signal compared to HeLa cell-free extract; however, as with HeLa cell-free extract and with the defined system, Gadd45 did not have a stimulatory effect on excision by the ML-1 cell-free extract (Fig. 3).

As Smith *et al.* (*1*) used the repair synthesis assay, and as they found that Gadd45

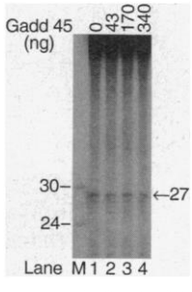

Fig. 1. Effect of Gadd45 on DNA repair by HeLa cell-free extract as measured by the excision assay. HeLa cell-free extract (50 μg) was supplemented with the indicated amounts of Gadd45 protein, and excision reaction was carried out under standard conditions (*5*) for 60 min. Human excinuclease excised the lesion in 25- to 27-nucleotide-long oligomers (*6*), which were resolved on 10% denaturing polyacrylamide gels. Lane M contains 30- and 24-nucleotide-long oligomers used as size markers. Arrow indicates the major excision product.

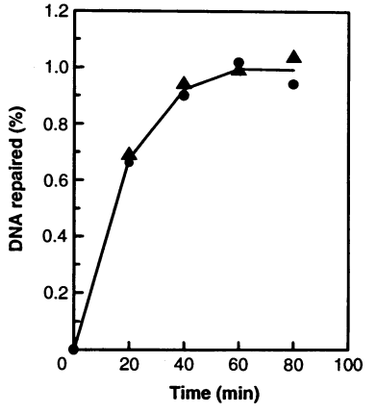

Fig. 2. Effect of Gadd45 on the kinetics of DNA excision repair with HeLa cell-free extracts. HeLa cell-free extracts (50 μg) were supplemented with 340 ng of Gadd45 protein, and the excision assay was carried out for the indicated times. The products were analyzed on a 10% denaturing polyacrylamide gel. The level of repair was determined by analysis of the excision gels using PhosphorImager (Molecular Dynamics, Sunnyvale, California). Data points are averages of two experiments. Circles, without Gadd45; triangles, with Gadd45.

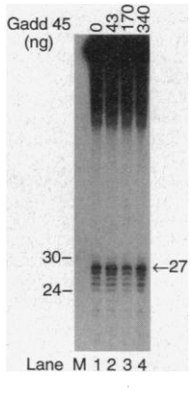

Fig. 3. Effect of Gadd45 on excision repair with ML-1 cell-free extracts. Indicated amounts of Gadd45 protein were added to ML-1 cell-free extracts (50 μg) and excision assay was performed under standard conditions (*5*).

binds to PCNA (proliferating cell nuclear antigen), which is involved in the repair synthesis step (*3*, *4*), we considered the possibility that repair synthesis might be stimulated by an unknown mechanism involving the Gadd45-PCNA complex. We conducted repair synthesis assay to test this possibility. Gadd45 had no effect on repair synthesis either. The experiment was repeated with the RKO cell-free extracts (another cell line used by Smith *et al.*), but we could not reproduce the results of Smith *et al.* We therefore conclude that Gadd45 does not modulate the activity of human excision nuclease, nor does it have an effect on the repair synthesis step of human nucleotide excision repair.

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5. HeLa S3 cells were from the stock of the Lineberger Comprehensive Cancer Center (University of North Carolina). ML-1 cell line and the RKO cell-free extracts were provided by A. Fornace and M. Smith. Cell-free extracts were prepared by the method of Manley *et al.* (*8*) and 50 μg were used for the excision or the repair synthesis assays. Substrate for the excision assay, which measures the excision of radiolabeled fragments carrying the lesions, was a 140-bp DNA containing cholesterol instead of a base in the center of one strand and a ^{32}P-radiolabel at the sixth phosphodiester bond 5′ to cholesterol (*2*). Approximately 1 nM substrate was incubated in the 25 μl reaction mixture containing 50 μg of cell-free extract; 40 mM Hepes, pH 7.9; 80 mM KCl; 40 mM NaCl; 8 mM MgCl; 2 mM ATP; 20 μM of each dNTP, 1 mM dithiothreitol, 3.5% (v/v) glycerol; and 100 μg bovine serum albumin at 30°C for 60 min. After incubation, the reaction mixtures were treated with proteinase K, phenol-chloroform extracted; the DNA was precipitated with ethanol and resolved on a 10% denaturing polyacrylamide gel. Excision assay in the defined system was carried out as described previously (*2*). The appearance of the ^{32}P-labeled fragments in 25- to 27-nucleotide range was taken as the measure of excinuclease activity. For the repair synthesis assay, pBR322 at 50 μg/ml in TE buffer was irradiated with 254 nm of ultraviolet (UV) light (400 J/m^2), which generated about 15 photolesions per molecule of DNA. Two hundred fifty nanograms of damaged or, as a control, undamaged DNA were used per assay. The repair synthesis reactions were carried out in 25 μl, as described by Smith *et al.* (*1*).
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Fornace and colleagues identified *GADD45* as an mRNA species with elevated expression in cells that have undergone growth arrest or DNA damage (*1*). The mRNA is induced by a wide range of stresses, including hypoxia, γ-irradiation, and genotoxic drugs such as etoposide (*2*). Overexpression of the 165 amino acid Gadd45 protein can induce growth arrest (*3*) and possibly apoptosis (*4*). Gadd45 binds to PCNA (*5*, *6*), a protein that participates in both semiconservative replication and nucleotide excision repair (NER) of damaged DNA (*7*). Because of the interaction with PCNA, Smith *et al.* inquired whether Gadd45 protein has an effect on NER and reported that addition of recombinant Gadd45 protein enhances DNA repair by nuclear extracts from human cells (*5*).

We investigated this possibility by adding recombinant glutathione-S-transferase (GST)–Gadd45 (*8*) to in vitro NER reactions (*9*). Repair of UV-damaged DNA mediated by whole cell extracts from a normal human fibroblast cell line was monitored (Fig. 1A). No effect was detected with the use of up to 16 μg of GST-Gadd45 per milliliter (lanes 1 to 3); minor variations between samples were also seen with GST control peptide (lanes 4 to 6). Addition of an anti-Gadd45 antibody also did not alter repair synthesis by the cell extract (lanes 8 and 9). Similarly, repair synthesis by HeLa cell extract was unaffected by either GST-Gadd45 or Gadd45 protein supplied by Smith and Fornace (*10*).

Although the assay for NER by repair synthesis is well established, it is subject to

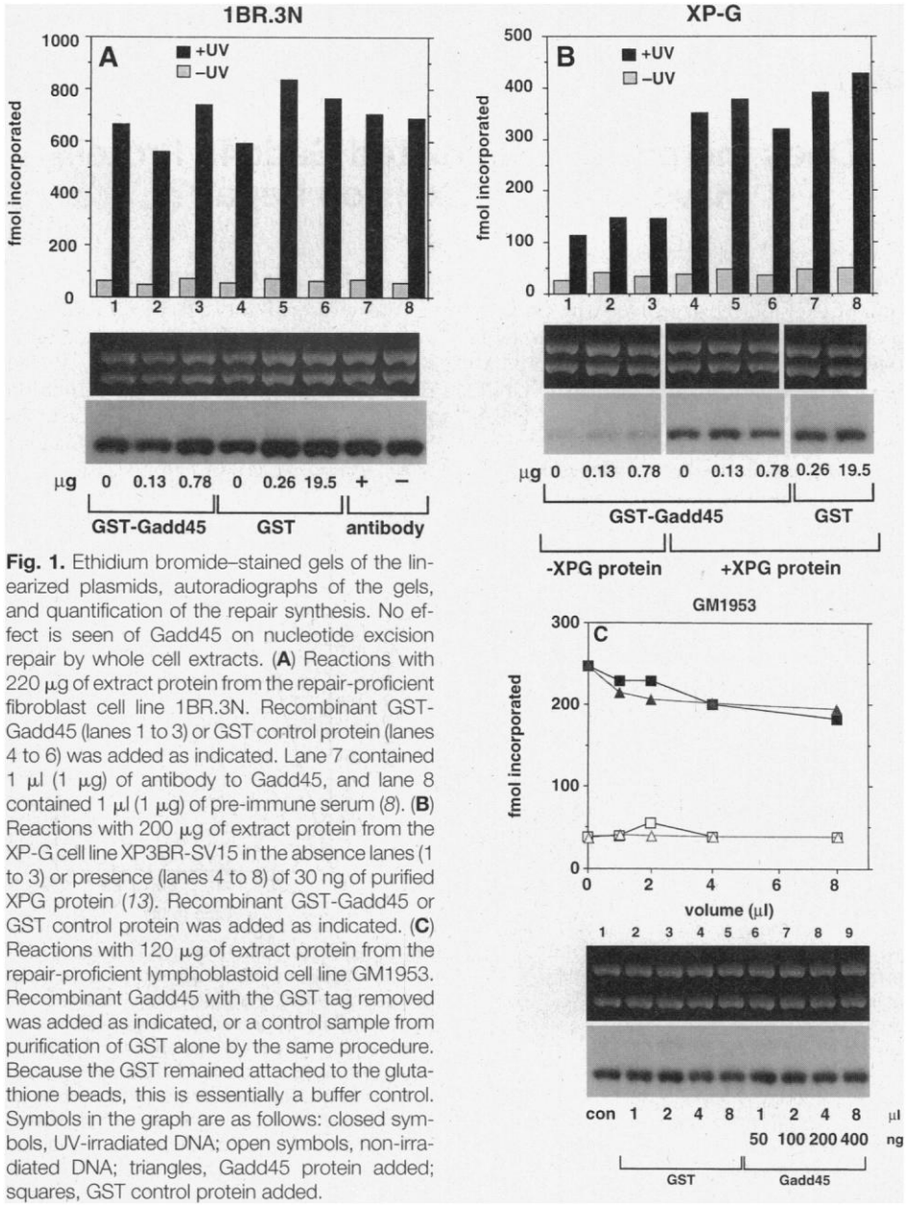

Fig. 1. Ethidium bromide–stained gels of the linearized plasmids, autoradiographs of the gels, and quantification of the repair synthesis. No effect is seen of Gadd45 on nucleotide excision repair by whole cell extracts. (**A**) Reactions with 220 μg of extract protein from the repair-proficient fibroblast cell line 1BR.3N. Recombinant GST-Gadd45 (lanes 1 to 3) or GST control protein (lanes 4 to 6) was added as indicated. Lane 7 contained 1 μl (1 μg) of antibody to Gadd45, and lane 8 contained 1 μl (1 μg) of pre-immune serum (*8*). (**B**) Reactions with 200 μg of extract protein from the XP-G cell line XP3BR-SV15 in the absence lanes (1 to 3) or presence (lanes 4 to 8) of 30 ng of purified XPG protein (*13*). Recombinant GST-Gadd45 or GST control protein was added as indicated. (**C**) Reactions with 120 μg of extract protein from the repair-proficient lymphoblastoid cell line GM1953. Recombinant Gadd45 with the GST tag removed was added as indicated, or a control sample from purification of GST alone by the same procedure. Because the GST remained attached to the glutathione beads, this is essentially a buffer control. Symbols in the graph are as follows: closed symbols, UV-irradiated DNA; open symbols, non-irradiated DNA; triangles, Gadd45 protein added; squares, GST control protein added.

some artifacts, and it is important to confirm that the damage-dependent repair synthesis observed is attributable to NER. We checked the results by the use of NER-defective xeroderma pigmentosum group G (XP-G) cell extracts, corrected by purified XPG protein. XP-G cell extract alone mediated a small amount of background synthesis that was unaffected by Gadd45 (Fig. 1B, lanes 1 to 3). The increase in repair after addition of XPG protein to the XP-G cell extract represents NER (compare lanes 1 and 4). Up to 16 μg of Gadd45 per milliliter did not affect this XPG-corrected signal (lanes 4 to 6), giving results similar to reactions where control GST protein was added (lanes 7 and 8). The GST-Gadd45 protein used in these experiments is functional, at least as defined by its binding to PCNA (6). To be sure that the GST tag was not interfering with any repair-enhancing effect of Gadd45, the fusion protein was cleaved with thrombin and repurified (8). Gadd45 purified in this manner also did not stimulate repair synthesis carried out by normal cell extract (Fig. 1C), but still bound to PCNA. Addition of Gadd45 slightly inhibited repair; apparently this was a buffer-related phenomenon, as a control sample in phosphate-buffered saline buffer had the same effect (Fig. 1C).

Gadd45 is only modestly induced by agents that stimulate NER, but Smith *et al.* (5) provided evidence that Gadd45 might be of fundamental importance in the interaction of cell cycle arrest and DNA repair. They found that addition of 0.8 to 8 μg of Gadd45 protein per milliliter gave a threefold stimulation of synthesis in UV-irradiated DNA by extracts from human cells. We have not been able to see such a stimulation of nucleotide excision repair with the use of extracts from cells with compromised p53 (pSVneo-immortalized fibroblasts) or from lymphoblastoid cells with normal p53 status. The reason for the variance from the results of Smith *et al.* (5) is uncertain. One difference appears to be that the repair synthesis observed in their study was quantitatively low (5), although a lack of absolute quantification prohibits a direct comparison with our results. A second difference is that nuclear extracts were used for repair synthesis, rather than the whole cell extracts used in our well-characterized system (*11*). We have so far been unable to find a nuclear extract procedure that reliably retains all of the protein components necessary for a full nucleotide excision repair reaction. In any case, the biological relevance of any stimulatory effect of Gadd45 on DNA nucleotide excision repair by means of an interaction with PCNA is uncertain, as damage recognition and incision of NER rather than repair DNA synthesis (*7*, *12*) are the rate-limiting steps.

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9. All methods were as described by Wood *et al.* (*11*). Whole cell extracts from the pSVneo immortalized fibroblast cell line 1BR.3N, the SV40-immortalized XP-G cell line XP3BR-SV15, and the normal lymphoblastoid cell line GM1953 had protein concentrations of 20 to 25 mg/ml. Reaction mixtures included the 3.0-kb pBluescript KS+ and the 3.7-kb pHM14 plasmids. pBluescript KS+ was UV-irradiated (450 Jm^{-2}). Both plasmids were treated with *E. coli* Nth protein, and closed-circular DNA was isolated from cesium chloride and sucrose gradients. Repair synthesis reactions were incubated at 30°C for 3 hours. DNA was purified and digested with *BamH*1 before electrophoresis.
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Response: We reported that the p53-regulated gene product Gadd45 binds to PCNA, a protein involved in DNA replication and repair (*1*). We also found that forced overexpression of *GADD45* resulted in growth inhibition, evidenced by delayed S-phase entry in transiently transfected cells. Finally, immunodepletion of Gadd45 from extracts of irradiated cells resulted in decreased DNA repair of damaged plasmids as measured by an in vitro assay, while addition of recombinant Gadd45 resulted in increased repair in this assay. Although the interaction of Gadd45 with PCNA and the growth inhibition by *GADD45* overexpression have been confirmed in other studies (*2*, *3*), the exact role of Gadd45 in DNA repair is less certain, on the basis of the findings of Kazantsev and Sancar and Kearsey *et al.*

Nucleotide excision repair is a complex process that involves damage recognition and excision, which are followed by repair synthesis (*1*, *4*, *5*). In order to determine at what stage Gadd45 acts, a collaboration with Sancar's group was initiated to study the earlier excision events. Because Sancar and his colleagues called our attention to their findings with the use of an excision-based assay and a repair synthesis assay, we have conducted additional experiments to help clarify the role of Gadd45 in DNA

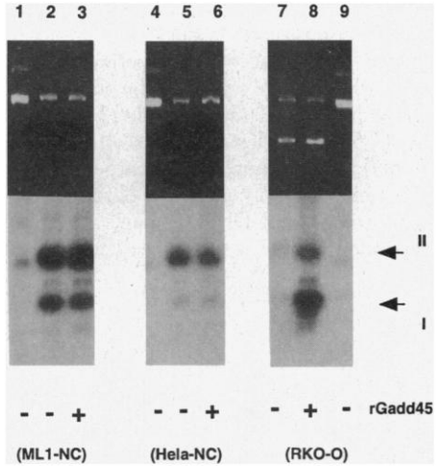

Fig. 1. Direct comparison of the two types of extract in the in vitro DNA repair synthesis assay. Panels shown are from an experiment of 19 May 1995. Lanes 1 to 6, whole cell extracts obtained from Sancar's laboratory, designated "NC" (North Carolina), either from ML1 (ML1-NC) or HeLa (HeLa-NC) cells; lanes 7 to 9, our (nuclear) extracts, prepared as previously described (*1*) (designated "O" for original), from RKO cells (RKO-O). Approximately 30 μg of extract proteins was used in each assay sample. Recombinant Gadd45 protein (100 ng) was added to the reactions where indicated (rGadd45); plasmid templates (1 μg) were either an undamaged control of larger size (lanes 1, 4, and 9) or contained AAAF adducts (all other lanes). In this experiment, plasmid templates were not linearized after repair incubations; arrows denote the positions of supercoiled (I) and nicked (II) forms of the plasmid. Pronounced retrieval of supercoiled plasmid appears in lane 8. Equivalent exposure times are shown for lanes 1 to 9.

repair. We obtained extracts from Sancar and compared them side-by-side with extracts prepared in our laboratory. In our report (*1*), we used extracts enriched in nuclear proteins, prepared by Dounce homogenization (*4*). Sancar's group uses whole cell extracts prepared by hypotonic lysis (*5*). Both types of crude extracts have been reported to carry out repair synthesis in vitro (*4*, *5*), although the latter (Manley-type extract) appears to be more widely used. We tested the effect of Gadd45 addition in the repair assay with both types of extract and a plasmid template damaged by N-acetoxy-2-acetylaminofluorene (AAAF), which produces a major type of adduct that is repaired by nucleotide excision repair. We agree with the comments that addition of rGadd45 has no effect when whole cell extracts are used. When our extracts were used, as prepared originally, modest stimulation of [^{32}P]dCTP incorporation was observed with addition of Gadd45 (Fig. 1). Incorporation was quantitated by direct counting with a Betagen Betascope (Waltham, Massachusetts), by densitometry, or both. We observed up to fourfold stimulation by rGadd45 addition with the use of nuclear extracts as a source of basal DNA repair activity, with values of 1.5-fold or greater in 15 out of 17 experiments (*6*). With whole cell extracts, on the other hand, the addition of Gadd45 yielded lower values (1.5-fold or less in 6 out of 6 experiments). Even though the incorporation of radioactivity that we have observed appears to be both DNA damage and ATP dependent, these results raise the issue of whether the stimulation we observed is a result of some activity other than nucleotide excision repair.

We conducted a series of experiments in which plasmids were not linearized by restriction enzyme digestion after the assay in order to visualize the condition of the template after the repair incubation. With our (nuclear) extracts, much of the input plasmid was retrieved in supercoiled form, and [^{32}P]dCTP incorporation stimulated by the addition of Gadd45 was observed primarily in the form of a supercoiled plasmid (Fig. 1, right panel). With (whole cell) extracts from Sancar's laboratory, the damaged plasmid was retrieved primarily in a nicked form (Fig. 1, left panels) and, in our hands, HeLa extracts consistently yielded approximately 50% lower incorporation than those from ML-1 prepared in the same manner. These observations raise the possibility that some component is present in the nuclear extracts, at a different concentration than in the whole cell extracts, which may be required for stimulation to be observed.

On the basis of the results of Kazantsev and Sancar and Kearsey *et al.*, particularly those for which purified components were used, we think it is likely that Gadd45 is not directly involved in the nucleotide excision event. Their findings do not exclude, however, an indirect contribution of Gadd45 to DNA repair. At least in yeast, corresponding Gadd45-interacting regions of the PCNA molecule (*2*), when mutated, increase UV sensitivity (*7*). In mammalian cells, defects in the p53 pathway are associated with sensitivity to DNA-damaging agents, including UV radiation (*8*), cisplatin (*9*), and mitomycin C (*10*), although it remains to be determined which of these observations may be a result of Gadd45 and other p53-regulated effector gene products, or even of the direct activities of p53 itself (*11*).

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