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trans-Dominant Inhibition of Poly(ADP-Ribosyl)ation Sensitizes Cells against γ-Irradiation and *N*-Methyl-*N'*-Nitro-*N*-Nitrosoguanidine but Does Not Limit DNA Replication of a Polyomavirus Replicon

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Poly(ADP-ribosyl)ation is a posttranslational modification of nuclear proteins catalyzed by poly(ADP-ribose) polymerase (PARP; EC 2.4.2.30), with NAD^+ serving as the substrate. PARP is strongly activated upon recognition of DNA strand breaks by its DNA-binding domain. Experiments with low-molecular-weight inhibitors of PARP have led to the view that PARP activity plays a role in DNA repair and possibly also in DNA replication, cell proliferation, and differentiation. Accumulating evidence for nonspecific inhibitor effects prompted us to develop a molecular genetic system to inhibit PARP in living cells, i.e., to overexpress selectively the DNA-binding domain of PARP as a dominant negative mutant. Here we report on a cell culture system which allows inducible, high-level expression of the DNA-binding domain. Induction of this domain leads to about 90% reduction of poly(ADP-ribose) accumulation after γ-irradiation and sensitizes cells to the cytotoxic effect of γ-irradiation and of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. In contrast, induction does not affect normal cellular proliferation or the replication of a transfected polyomavirus replicon. Thus, *trans*-dominant inhibition of the poly(ADP-ribose) accumulation occurring after γ-irradiation or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine is specifically associated with a disturbance of the cellular recovery from the inflicted damage.

One of the most rapid responses of eukaryotic cells to DNA damage is poly(ADP-ribosyl)ation of proteins, a posttranslational modification catalyzed by the highly abundant nuclear enzyme poly(ADP-ribose) polymerase (PARP; EC 2.4.2.30), which uses NAD^+ as a substrate (for reviews, see references 4, 9, and 20). While PARP itself serves as the main acceptor of poly(ADP-ribose), other chromosomal proteins such as histones (41) and topoisomerases I (42) and II (68) have been shown to be modified as well. PARP has a domain structure: its DNA-binding domain (DBD), located at the amino terminus, specifically binds to DNA single- or double-strand breaks. Binding to strand breaks is mediated by two zinc fingers and causes a drastic activation of the catalytic center within the carboxy-terminal NAD^+-binding domain. Recently, the catalytic function was attributed to dimerized PARP (52). PARP automodification is thought to preferentially occur in a separate protein domain, located between the DBD and the NAD^+-binding domain, although the latter domains also may be poly(ADP-ribosyl)ated (21). Treatment of cells with chemical or physical agents that cause DNA strand breaks induces a dose-dependent stimulation of poly(ADP-ribose) synthesis. High polymer concentrations, in turn, stimulate poly(ADP-ribose) glycohydrolase, the main polymer-degrading enzyme, leading to rapid polymer turnover (5). cDNA sequences of PARP from humans (19, 44, 75), mice (30), rats (72), cattle (64), *Xenopus laevis* and cherry salmon (59), and *Drosophila melanogaster* (74) have been determined, revealing an extensive interspecies homology that culminates in the region surrounding the catalytic center of the NAD^+-binding domain (20).

To elucidate possible biological functions of poly(ADP-ribosyl)ation, NAD^+ analogs (benzamide and derivatives) have often been used as competitive PARP inhibitors. At concentrations of 1 mM or lower, these classical inhibitors have little influence on cell proliferation, nor are they mutagenic or carcinogenic, but they potentiate the cytotoxicity and chromosomal damage induced by alkylating agents, ionizing radiation, and other DNA-damaging treatments. These and other findings led to the view that poly(ADP-ribosyl)ation plays a role in DNA repair (24) and in other cellular responses to DNA damage, such as cell cycle perturbations (34, 35), DNA amplification (15, 14, 28), malignant transformation (8, 37, 71), and, under conditions of extensive DNA breakage and PARP activation, cell death (7, 61, 78). In addition, some evidence has suggested that poly(ADP-ribosyl)ation is involved in DNA replication, cell differentiation, integration of foreign DNA into the cell genome, and intrachromosomal homologous recombination (for reviews, see references 4, 10, and 20). It should be noted, however, that there are three classes of ADP-ribosyltransferases with different levels of sensitivity to ADP-ribosylation inhibitors (62), and nonspecific inhibitor effects have been noted (53, 54, 57). Thus, it is difficult to draw definitive conclusions for biological functions of PARP on the basis of inhibitor experiments alone.

To overcome this problem, we have developed a molecular genetic approach to modulate poly(ADP-ribose) metabolism in living cells, i.e., the *trans*-dominant inhibition of poly(ADP-ribosyl)ation by overexpression of the 42-kDa DBD of human

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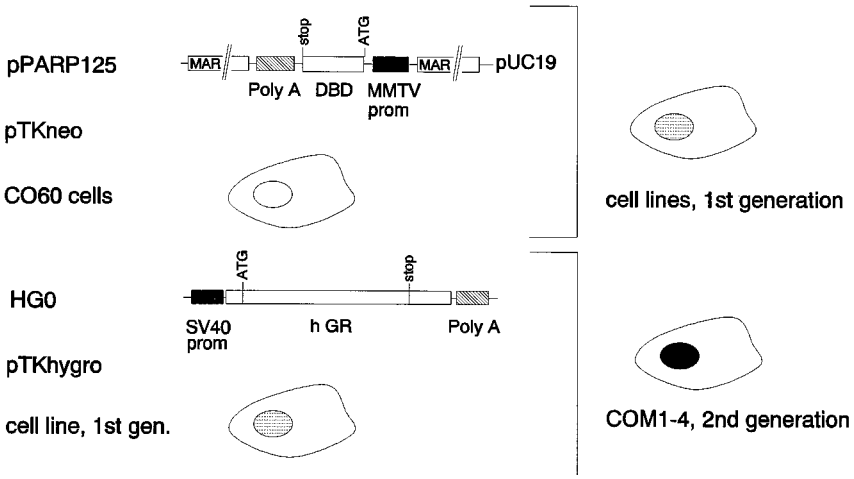

FIG. 1. Generation of stably transfected cell lines inducibly overexpressing the PARP DBD. Plasmid pPARP125 harbors the coding sequence of the PARP DBD under the control of the MMTV long terminal repeat (MMTV Prom, central *Hae*III fragment). The expression cassette is flanked by an inverted duplicate of the chicken lysozyme 5′ upstream matrix attachment region element (MAR) (2.95-kb *Bam*HI-*Xba*I fragment). Plasmid pPARP125 was cotransfected into CO60 hamster cells together with pTKneo, a plasmid harboring the neomycin resistance gene under the control of the herpes simplex virus thymidine kinase gene promoter, resulting in first-generation cell lines. In plasmid HG0, the human glucocorticoid receptor cDNA (h GR) had been inserted into the expression vector pKCR2 (11) harboring the SV40 early promoter (SV40 prom). Plasmid HG0 was cotransfected into one of the first-generation cell lines together with pTKhygro, a plasmid harboring the hygromycin resistance gene driven by the thymidine kinase promoter, resulting in the second-generation cell lines (COM1 to COM4).

PARP as was shown in transient transfection assays (43, 55). Here we show the following. (i) A high level of DBD expression is achievable in stably transfected cell lines. (ii) The resulting *trans*-dominant inhibition of poly(ADP-ribosyl)ation is about 90%. (iii) Neither the rate of normal cellular proliferation nor the episomal DNA replication of a transfected polyomavirus replicon is affected by this DBD overexpression. (iv) In contrast, cell survival following γ-irradiation or *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) is specifically and substantially reduced when the DBD is overexpressed at the time of irradiation.

MATERIALS AND METHODS

Antibodies and plasmids. Anti-FII serum (rabbit) raised against the second zinc finger of PARP (69) was kindly provided by G. de Murcia, Strasbourg, France. Mouse monoclonal antibody 10H (38) raised against poly(ADP-ribose) was kindly provided by M. Miwa and T. Sugimura, Tokyo, Japan. Polyclonal anti-human glucocorticoid receptor antibody (anti-amino acids 346 to 367) was from Dianova, Hamburg, Germany. Phosphatase-conjugated goat anti-rabbit immunoglobulins were from Sigma, Munich, Germany. Cy3-conjugated donkey anti-rabbit immunoglobulins were from Dianova. Fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulins were from Renner, Dannstadt, Germany. Human glucocorticoid receptor expression construct HG0 was kindly provided by P. Chambon and H. Gronemeyer, Strasbourg, France. Plasmid vector with flanking DNA of the chicken lysozyme matrix attachment region was kindly provided by W. H. Strätling, Hamburg, Germany. Plasmid pTKneo was kindly provided by M. Boshart, Munich, Germany.

Cell culture. The simian virus 40 (SV40)-transformed Chinese hamster cell line CO60 (46), kindly provided by S. Lavi, Tel Aviv, Israel, was maintained as monolayer in routine medium, i.e., Dulbecco modified Eagle medium (GIBCO, Karlsruhe, Germany) supplemented with L-glutamine (1 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 5% fetal calf serum (GIBCO). Cells were free of *Mycoplasma* contamination. Stable transfectants of CO60 were propagated in routine medium supplemented with the following: (i) 800 μg of geneticin (GIBCO) per ml for COC cell clones; (ii) 800 μg of geneticin per ml plus 800 U of hygromycin B (Calbiochem, Bad Soden, Germany) per ml for COM cell clones; and (iii) 800 U of hygromycin B per ml for COR cell clones. Selection media were replaced by routine medium 1 day prior to dexamethasone (Dex) treatment, which was performed for 24 h. Therefore, at the starting points of any experimental manipulations (irradiations, MNNG treatment, electrotransfection, etc.), all cell lines were off selection antibiotics for 2 days.

Stable transfection of cells. For the generation of COM cell lines (Fig. 1), semiconfluent CO60 cultures on 10-cm-diameter petri dishes were transfected with 18 μg of pPARP125 plus 2 μg of pTKneo by the calcium phosphate method (76). Cells were selected with 800 μg of geneticin per ml, and resistant clones were isolated and analyzed by Western blotting (immunoblotting). One of the clones (pPARP125) (Fig. 1) with inducible DBD expression was supertransfected with HG0 and pTKhygro. Colonies resistant to 800 U of hygromycin B per ml were isolated and analyzed by Western blotting and immunofluorescence. A number of strongly expressing clones were subcloned by endpoint dilution.

For the generation of control cell lines overexpressing the human glucocorticoid receptor only (COR cells), CO60 cells were transfected with 18 μg of HG0 plus 2 μg of pTKhygro, using the same transfection protocol. Colonies resistant to 800 U of hygromycin B per ml were isolated and screened by Northern (RNA) blotting (51), using human glucocorticoid receptor cDNA as a probe. A number of positive clones were subcloned by endpoint dilution.

Cell survival. Cell survival was assessed by using the following protocols. (i) Exponentially growing cell monolayers were subjected to either γ-irradiation (^{137}Cs-Gamma Cell 1000, dose rate of 14 Gy · min^{-1}; Atomic Energy of Canada Limited) in phosphate-buffered saline (PBS; 137 mM sodium chloride, 2.7 mM potassium chloride, 1.5 mM potassium dihydrogen phosphate, 8.1 mM disodium hydrogen phosphate) or to UV-C irradiation (germicidal lamp emitting predominantly at 253.7 nm with a fluence rate of 0.1 J · m^{-2} · s^{-1}) after rinsing with PBS at room temperature. After irradiation, medium was added and cells were allowed to repair DNA damages for 16 h under routine cell culture conditions. Then cells were trypsinized and replated onto 10-cm-diameter petri dishes. Eight to ten days later, colonies were fixed with 10% formaldehyde in PBS and stained with 0.1% crystal violet. The number of colonies consisting of more than 50 cells was determined.

(ii) To assess survival following MNNG treatment, a modified procedure was used. Exponentially growing cells were trypsinized, counted, and seeded onto 10-cm-diameter petri dishes in routine medium. Six hours later, the medium was replaced with routine medium supplemented with MNNG (Serva). Cells were further incubated in this medium for 10 days. Colonies were fixed as described above.

Electrotransfection. Exponentially growing cells were trypsinized, and 5×10^6 cells were resuspended in 140 μl of PBS and combined with 20 μg of supercoiled plasmid DNA dissolved in 60 μl of TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA). Electrotransfection (Gene Pulser and capacitance extender; Bio-Rad Laboratories, Richmond, Calif.) was done at 140 V and 960 μF in cuvettes of 0.2-cm electrode distance (Bio-Rad). Ten minutes later, cells were resuspended in medium and dispensed into culture flasks.

Indirect immunofluorescence. Cells grown on coverslips were subjected to 56 Gy of γ-irradiation in PBS at room temperature. Five minutes after the onset of irradiation cells were fixed with 10% ice-cold trichloroacetic acid as described earlier (43). For double-immunofluorescence analysis, coverslips were incubated with a first-antibody mixture consisting of the anti-FII serum (diluted 1:200 in PBS–1% bovine serum albumin [BSA]) and monoclonal antibody 10H (concentrated 10-fold by ultrafiltration of hybridoma supernatant) for 45 min at 37°C. Coverslips were incubated with the second-antibody mixture consisting of Cy3-conjugated anti-rabbit immunoglobulins (diluted 1:100 in PBS–1% BSA) and fluorescein isothiocyanate-conjugated anti-mouse immunoglobulins (diluted 1:50 in PBS–1% BSA) for 30 min at 37°C. Immunofluorescence was evaluated with a Leitz Dialux 22 EB microscope (Leitz, Wetzlar, Germany).

Western blotting. To prepare crude protein extracts, cells were trypsinized, washed once in PBS supplemented with protease inhibitors (10 mM sodium bisulfite, 10 μM pepstatin, 1 mM phenylmethylsulfonyl fluoride), and resuspended in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer. After separation by SDS-PAGE (10% polyacrylamide gel) (45), proteins were electroblotted onto Immobilon-P membranes (Millipore, Eschborn, Germany). Blots were incubated overnight at 4°C with the anti-FII serum diluted 1:2,000 in PBS–Tween–5% dry milk or with the anti-human glucocorticoid receptor antibody diluted 1:1,000. Blots were developed with phosphatase-conjugated goat anti-rabbit immunoglobulins.

Southern blotting. Extraction of total DNA from cells was performed as described previously (51). Six micrograms of total DNA of each sample was separated in a 0.7% agarose gel and blotted onto Gene Screen membranes (Du Pont, Bad Nauheim, Germany) as described previously (70). The membranes were hybridized in a mixture containing 0.9 M NaCl–0.09 M sodium citrate (pH 7.0), 5× Denhardt's solution, 0.1 mg of tRNA per ml, and 1% SDS at 42°C with ^{32}P-labeled oligonucleotide py6-26 (5′-CCCCTGGCCTCCGCTTACTCT-3′) recognizing the replication origin of polyomavirus strain A2. The membranes were washed in 0.9 M NaCl at 56°C and exposed to Kodak X-Omat AR films.

Quantitation of poly(ADP-ribose) levels in living cells. Radioactive labeling of cells with [^{3}H]adenine (ICN Biomedicals, Meckenheim, Germany) and purification of NAD^+ and poly(ADP-ribose) by using affinity column chromatography with dihydroxyboronyl-Bio-Rex 70 (DHB-B) were performed as described previously (1). To determine the specific radioactivity of the NAD^+ pool, the radioactivity of purified NAD^+ was determined by liquid scintillation counting, while the absolute amount of NAD^+ was determined with a NAD^+ cycling assay (33). The absolute amount of purified poly(ADP-ribose) was calculated from its radioactivity and the specific activity of the NAD^+ pool.

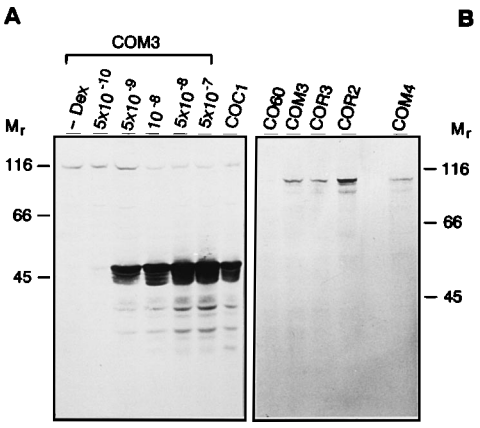

FIG. 2. Western blot analysis of PARP DBD and glucocorticoid receptor expression. Extracts from 10^5 cells were separated on SDS–10% polyacrylamide gels and transferred onto Immobilon-P membranes as described in Materials and Methods. (A) COM3 cells were incubated for 24 h with Dex at the concentrations indicated (moles per liter). Additionally, an extract of COC1 cells overexpressing the PARP DBD constitutively was loaded. The blot was probed with the anti-FII serum, recognizing the PARP DBD. (B) Extracts from the cell lines indicated were probed with an anti-human glucocorticoid receptor antiserum. Relative molecular weights (in thousands) are indicated.

RESULTS

Generation of stably transfected cell lines expressing the PARP DBD and of control lines. Selective overexpression of the PARP DBD leads to *trans*-dominant inhibition of poly(ADP-ribosyl)ation, as was demonstrated in transient transfection experiments (43, 55). To precisely quantitate this inhibition and to obtain further insight into the biological role(s) of poly(ADP-ribosyl)ation, we set out to establish stably transfected cell lines. Three sets of transfection experiments were done.

(i) For the generation of cell lines with constitutive overexpression of the DBD, pPARP6 (43), a construct carrying the DBD under the control of the human cytomegalovirus promoter/enhancer, was cotransfected with the neomycin resistance gene into CO60 hamster cells. G418-resistant colonies were isolated and screened by Western blotting with an antiserum (anti-FII) directed against the second zinc finger of the DBD (data not shown). Two positive clones were expanded. The Western blot shown in Fig. 2A reveals that clone COC1 expressed the 42-kDa DBD in vast excess over the resident 113-kDa PARP (note that in SDS gels, PARP migrates at 116 kDa and the DBD migrates at about 46 kDa, while the predicted molecular masses are 113,153 and 41,968, respectively [44]).

(ii) To obtain Dex-inducible expression of the PARP DBD, we chose the central *Hae*III fragment of the mouse mammary tumor virus (MMTV) long terminal repeat (26) as a promoter sequence. In the plasmid construct generated (pPARP125), the expression cassette is flanked by an inverted duplicate of the chicken lysozyme 5′ upstream matrix attachment region element (Fig. 1). Such sequences are thought to confer copy number-dependent expression from heterologous promoters independent of the chromosomal integration site (39, 60). Cotransfection of this construct with a neomycin resistance gene resulted in first-generation cell lines. Although Dex-inducible expression was detectable on Western blots in a number of these clones, the levels of DBD expression were not sufficient to inhibit resident PARP activity significantly when analyzed by double immunofluorescence (data not shown). The lack of efficient DBD expression turned out to be due to a relatively low level of the glucocorticoid receptor in the parental cell line CO60 (Fig. 2B), as had been shown for a number of other cell lines (2, 6, 27, 40). Thus, we supertransfected a construct for the constitutive expression of the human glucocorticoid receptor (HG0; Fig. 1) together with a selection marker conferring resistance to hygromycin B into one of the first-generation cell lines. By hygromycin selection, second-generation cell lines (Fig. 1) were obtained. Southern blot analysis of the glucocorticoid receptor cDNA integration patterns revealed that four of the lines tested were of independent clonal origin (data not shown). These were subcloned to give rise to clones COM1 to COM4. Figure 2A reveals that in subclone COM3, the 42-kDa DBD was drastically induced with as little as 5×10^{-9} M Dex, whereas in uninduced cells, no DBD expression was detectable under these conditions. The same results were obtained with the other COM subclones (data not shown). Immunoreactive material migrating faster than the main DBD band was of variable abundance in different experiments and is most likely due to proteolytic degradation.

(iii) To generate control cell lines that overexpress the glucocorticoid receptor only, plasmid HG0 was cotransfected with the hygromycin resistance gene into CO60 hamster cells. Hygromycin-resistant clones were isolated and screened by Northern and Western blotting (data not shown). Two positive clones were subcloned, giving rise to COR2 and COR3. Western blot analysis (Fig. 2B) revealed that the amounts of human glucocorticoid receptor were comparable in COM3 and COR3 and about fivefold higher in COR2. The endogenous receptor of CO60 parental cells was barely detectable by Western blotting. (The anti-glucocorticoid receptor antiserum recognizes receptor of human, mouse, and rat origin [supplier's information].)

Analysis of PARP activity in stably transfected cell lines. The effect of DBD overexpression on cellular poly(ADP-ribosyl)ation was investigated by both double immunofluorescence (43) and in vivo radiolabeling of poly(ADP-ribose) (1). Double immunofluorescence allows for the determination of the percentage of cells that express the foreign DBD gene and at the same time yields an estimate for the cellular poly(ADP-ribose) formation. A representative example of a double-immunofluorescence analysis of PARP activity done in COM3 cells is given in Fig. 3. By using our current immunofluorescence protocol,

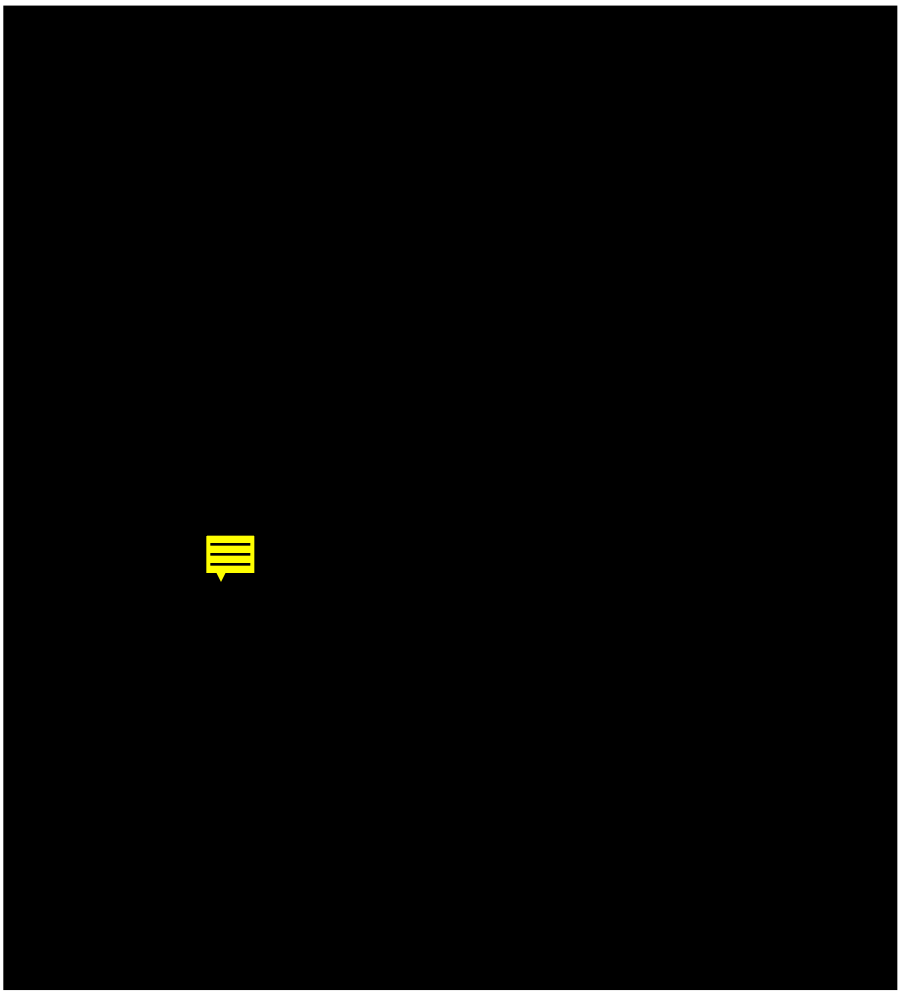

FIG. 3. Detection of DBD expression and poly(ADP-ribose) formation in living cells by double immunofluorescence. COM3 cells growing on coverslips were induced with Dex (5×10^{-8} M) or left uninduced for 24 h. Cells were γ-irradiated (56 Gy) or not in PBS as indicated and processed for double immunofluorescence, using the rabbit anti-FII serum against the DBD and mouse monoclonal antibody 10H against poly(ADP-ribose). The secondary antibody recognizing the anti-FII serum was labeled with Cy3 (red), while the one recognizing 10H was labeled with fluorescein isothiocyanate (green). Identical microscopic fields were photographed.

we were unable to detect the very low basal levels of poly(ADP-ribose) in cultured cells, which is possible by using a high-pressure liquid chromatography method (36). However, when cells are treated with a DNA-damaging agent like γ-irradiation to stimulate PARP, polymer immunofluorescence signals that are characterized by a fine-granular pattern visible under high magnification (12) and can be distinguished from the nonspecific staining of nucleoli are readily obtained. Accordingly, in unirradiated COM3 cells, nuclear polymer-specific staining was not detectable (Fig. 3) regardless of whether the DBD had been induced by Dex treatment. (Typically, Dex treatment led to DBD immunofluorescence signals in about 95% of the cell population, while 5% of COM3 cells failed to express the DBD for unknown reasons.) The cytoplasmic staining in both fluorescence channels is nonspecific and of variable intensity because of automatic photographic exposure. In contrast, when uninduced cells were treated with high-dose γ-irradiation (56 Gy), all of the nuclei exhibited a brilliant polymer-specific staining. If the cultures had been Dex induced, these fluorescence signals were completely suppressed in all the cells that overexpressed the DBD, as was expected from our earlier findings in transient transfection experiments (43). Dex treatment of COR3 control cells had no detectable influence on polymer formation (data not shown).

To precisely quantitate the inhibitory effect on poly(ADP-ribose) accumulation, the technique of in vivo radiolabeling of poly(ADP-ribose) (1) was used. Cells readily convert ^{3}H-labeled adenine added to the medium to NAD^+, and PARP, if activated by DNA strand breaks, incorporates the label into poly(ADP-ribose). NAD^+ and poly(ADP-ribose), both radioactively labeled, are purified from cells and quantitated as described in Materials and Methods. The results of a typical in vivo radiolabeling experiment done with COM3 cells are depicted in Fig. 4. γ-Irradiation (56 Gy) stimulated PARP activity in uninduced COM3 cells drastically, leading to levels of about 20 pmol of poly(ADP-ribose) per 10^6 cells at 15 min after irradiation. Dex-induced cultures overexpressing the DBD exhibited about 90% inhibition of polymer accumulation. This is a conservative estimate, since no background subtraction was performed. Statistical analysis revealed that the residual polymer values were not significantly different from the values for unirradiated control cells or uninduced cells irradiated in the presence of 3-aminobenzamide (Fig. 4), a widely used chemical ADP-ribosylation inhibitor ($P > 0.05$ by Student's test). The

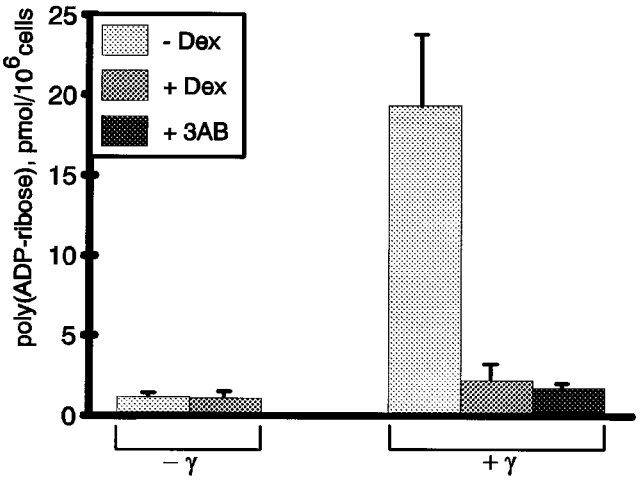

FIG. 4. Quantitative determination of poly(ADP-ribose) in [^{3}H]adenine-labeled COM3 cells. Uninduced cells or cells induced with Dex (5×10^{-8} M for 24 h), whose NAD^+ pools were labeled by preincubation of cells in [^{3}H]adenine-supplemented culture medium, were irradiated (56 Gy) or not in PBS. Poly(ADP-ribose) was purified from trichloroacetic acid-insoluble extracts by boronate chromatography and quantitated by liquid scintillation counting. Absolute amounts of poly(ADP-ribose) were calculated from the specific radioactivity of the NAD^+ pools as described in Materials and Methods. Assays were done in quadruplicate. Correction was performed for the 5% of cells that failed to express the DBD. Standard deviations are indicated. 3AB, uninduced cells were given 2 mM 3-aminobenzamide 2 h prior to irradiation.

specific radioactivities of the NAD^+ pools were the same in uninduced and induced cells (data not shown).

Biological consequences of DBD overexpression. Since poly(ADP-ribosyl)ation has been claimed to play a role in the cellular response to DNA damage as well as in normal cell physiology, we have investigated biological consequences associated with *trans*-dominant PARP inhibition both upon normal cell growth and following DNA damage.

First, we studied whether DBD overexpression had any influence on cell proliferation. Figure 5 shows that in COM3 as well as in the parallel clone COM4, induction of the DBD with 10^{-8} M Dex was associated with a slight prolongation of cell doubling time which was further increased at 5×10^{-8} M Dex in COM3. However, a similar response to Dex treatment was also noted in the COC1 cell line that constitutively overexpresses the PARP DBD, in the parental CO60 cell line, and in the glucocorticoid receptor-expressing control cell line COR3. Since it is known that Dex can lead to downregulation of glucorticoid receptor expression, depending on ligand concentration (16, 17, 58, 63), we verified by Western blotting and immunofluorescence that overexpression of the DBD and consequent PARP inhibition were indeed maintained in COM3 cells throughout the duration of the experiment (data not shown). Taken together, the data clearly show that the observed marginal cell growth retardation following Dex treatment was not a consequence of DBD overexpression. Most likely, it was due to the action of the glucocorticoid receptor, since COR2, the control cell line with the strongest receptor expression (Fig. 2B), also displayed the most pronounced growth retardation after Dex treatment (Fig. 5).

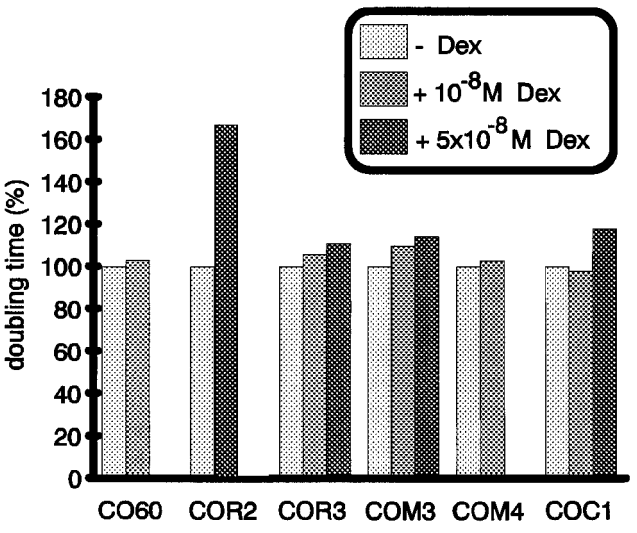

FIG. 5. Influence of Dex treatment on the doubling time of CO60 cells and derived cell clones. Cell lines were grown in the presence or absence of Dex as indicated. Cell proliferation was assayed at least in triplicate and is expressed as percentage of the doubling time of uninduced controls. In all cases, the relative standard deviation was equal to or less than 6.1%.

To assess more directly any influence of the overexpressed DBD on DNA replication, we supertransfected the shuttle plasmid pYZ289 into the COM3 cell line. The presence of the mouse polyomavirus replicon enables this plasmid to replicate episomally in mouse cells (56). Figure 6 shows a Southern blot that was probed with a ^{32}P-labeled oligonucleotide hybridizing with the polyomavirus origin of replication. It is evident that in COM3 hamster cells, too, massive DNA replication of the shuttle plasmid occurred upon electrotransfection, up to a level of about 10,000 copies per cell, as detected by the appearance of *Dpn*I-resistant and *Mbo*I-sensitive plasmid DNA. (Restriction endonuclease *Dpn*I digests nonreplicated input plasmid DNA, carrying the bacterial methylation pattern, but not DNA replicated in eukaryotic cells. For the isoschizomer *Mbo*I, the opposite applies.) As is evident from Fig. 6, eukaryotic DNA replication of the shuttle plasmid occurred irrespective of Dex induction of the DBD (note that in the lane +Dex/*Dpn*I, somewhat less DNA was loaded onto the gel). To verify the sustained inhibition of poly(ADP-ribosyl)ation, cells seeded in parallel onto coverslips were treated with 56 Gy of γ-irradiation and subjected to immunofluorescence. Poly(ADP-ribosyl)ation was continuously blocked when cells pretreated with Dex (5×10^{-8} M) were electrotransfected, grown in Dex-free medium for 16 h, and then again grown in Dex medium until 48 h posttransfection (data not shown). This induction protocol was chosen, since the continued presence of Dex following electrotransfection resulted in a higher cytotoxicity of the electroshock than in uninduced controls (data not shown). Taken together, the data show that overexpression of the DBD did not limit episomal DNA replication of the transfected shuttle plasmid.

A further biological endpoint that we studied was cell survival following DNA damage. Figure 7 shows the influence of Dex treatment on the survival of the COR3 glucocorticoid receptor-expressing cells and of COM3 cells following exposure to γ-rays, MNNG, or UV-C. The survival curves of noninduced COM3 and COR3 cells are not identical, reflecting some clonal variation in the intrinsic sensitivity between these cell lines. However, the actual effect of Dex is assessed within the two cell systems, with the uninduced cultures yielding the respective baseline. Treatment of the COR3 cell line with Dex (5×10^{-8} M) prior to γ-irradiation resulted in improved cell survival compared with uninduced cells (Fig. 7A; the protection factor is 1.3 at 37% survival [D_{37}]). This was also the case with the glucocorticoid receptor cell line COR2, which is clonally independent of COR3 (data not shown). By contrast,

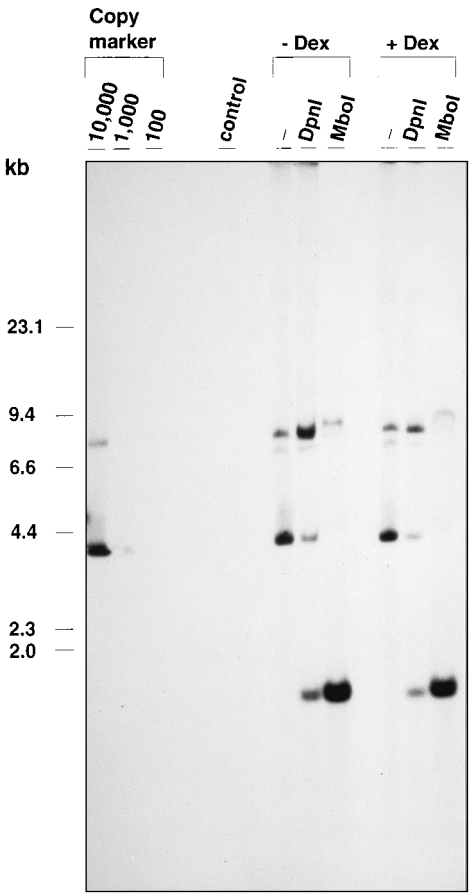

FIG. 6. Effect of DBD overexpression on episomal DNA replication. COM3 cells were induced with 5×10^{-8} M Dex or left uninduced for 24 h as indicated. Cells were supertransfected by electroporation with shuttle plasmid pYZ289 comprising the polyomavirus replicon. After electroporation, Dex-pretreated COM3 cells were incubated for 16 h in Dex-free medium and then again in Dex-supplemented medium to guarantee continued inhibition of poly(ADP-ribosyl)ation. Forty-eight hours after electroporation, total cellular DNA was extracted and digested with restriction endonuclease *Xho*I (noncutting enzyme for pYZ289), *Xho*I-*Dpn*I, or *Xho*I-*Mbo*I as indicated. *Xho*I-restricted DNA of untransfected COM3 cells served as a control. Unrestricted pYZ289 plasmid DNA was used as a copy marker (the position of supercoiled plasmid is at about 4 kb). Six micrograms of cellular DNA per lane was subjected to Southern blotting and hybridization with ^{32}P-labeled oligonucleotide py6-26, recognizing the polyomavirus origin of replication.

pretreatment of the COM3 cells with Dex (5×10^{-8} M) prior to γ-irradiation led to a clear-cut radiosensitization (Fig. 7B). In the representative experiment depicted, the cytotoxicity was potentiated by a factor of 1.7 at D_{37}. Dex pretreatment at a lower concentration (10^{-8} M) yielded virtually identical results (Fig. 7C). Thus, in DBD-overexpressing cells, the cytotoxicity was potentiated despite a protective effect of Dex treatment per se which was apparent in control cells and was probably mediated by the glucocorticoid receptor overexpression.

Dex treatment did not influence the survival of COR3 control cells following exposure to the alkylating agent MNNG (Fig. 7D), in contrast to the situation following γ-irradiation, but again greatly reduced the survival of COM3 cells (Fig. 7E; the sensitization factor at D_{37} is 4.2).

Finally, we were interested in whether the cell sensitization by DBD overexpression was dependent on the type of DNA damage inflicted to the cells. While both γ-irradiation and MNNG are known to induce DNA lesions that stimulate base excision repair, UV-C radiation causes the formation of a different class of DNA damages that is repaired mainly by nucleotide excision repair (66). Figure 7F reveals that in COR3 control cells, Dex pretreatment led to some protection against UV-C that was not apparent in COM3 cells overexpressing the DBD (Fig. 7G). In contrast to γ-irradiation and MNNG treatment, the survival curves of UV-C-treated cells had a sigmoid-like shape (Fig. 7F and G), which may be explained by inadequate irradiation of the cells in the periphery of the petri dishes. Taken together, the data show that inhibition of poly(ADP-ribosyl)ation was associated with only minor sensitization against UV-C irradiation, just balancing out the protective effect of Dex in our cell system.

DISCUSSION

We have established a molecular genetic approach to inhibit poly(ADP-ribose) formation in a *trans*-dominant fashion by overexpressing selectively the PARP DBD in cultured cells. This inhibition is due to the competition for DNA strand breaks by the overexpressed DBD against resident PARP (43). To study the biological consequences of enzyme inhibition, we generated stably transfected cell lines by two different approaches. First, we used plasmid pPARP6 to express the DBD of human PARP constitutively in stable transfectants of the hamster cell line CO60. Second, we set out to generate cell lines with Dex-inducible expression of the PARP DBD mediated by a fragment of the MMTV long terminal repeat (26). To further increase the expression of the DBD, we supertransfected an expression construct for the human glucocorticoid receptor into one of our first-generation cell lines (Fig. 1). Western blot and immunofluorescence experiments on the resulting second-generation cell lines (COM cells) revealed that the DBD was induced drastically in about 95% of the cell population with as little as 10^{-8} M Dex, while there was no detectable expression in the absence of Dex (Fig. 2 and 3). An advantage of the Dex-hyperresponsive cell culture system was that low-dose Dex treatment (1×10^{-8} to 5×10^{-8} M) that effectively induced DBD overexpression did not lead to a general downregulation of DBD expression even when administered over a period of several days, in contrast to treatment with 10^{-6} M Dex (data not shown), a concentration often used in cell lines for MMTV promoter-driven expression of transfected genes.

The effect of DBD overexpression on poly(ADP-ribosyl)ation was studied both by double immunofluorescence as an in situ technique for the simultaneous detection of the DBD and poly(ADP-ribose) and by in vivo radiolabeling of cells (Fig. 3 and 4). We could show that a strong inhibitory effect (about 90%) on resident PARP activity was apparent in all cells that overexpressed the DBD. Thus, the results of two assays are in good agreement and further confirm in stably transfected cells our previous results from transient transfections and double-immunofluorescence analyses (43, 55).

By expressing PARP antisense RNA under the control of the MMTV promoter in HeLa cells, Ding et al. (22) were able to reduce the level of cellular PARP activity by about 80% when their stable HeLa transfectant had been incubated with 10^{-6} M Dex for 72 h. This long induction time was necessary because of the relatively long half-life of PARP, ranging from 48 to 72 h. Dex induction for 72 h led to a growth retardation more pronounced than that observed in Dex-treated control cells. Furthermore, isolated nuclei of their Dex-pretreated transfectant were more sensitive to DNase I. Finally, the cells displayed a marked delay in the repair of single-stranded DNA breaks and were sensitized to the cytotoxic effects of alkylating

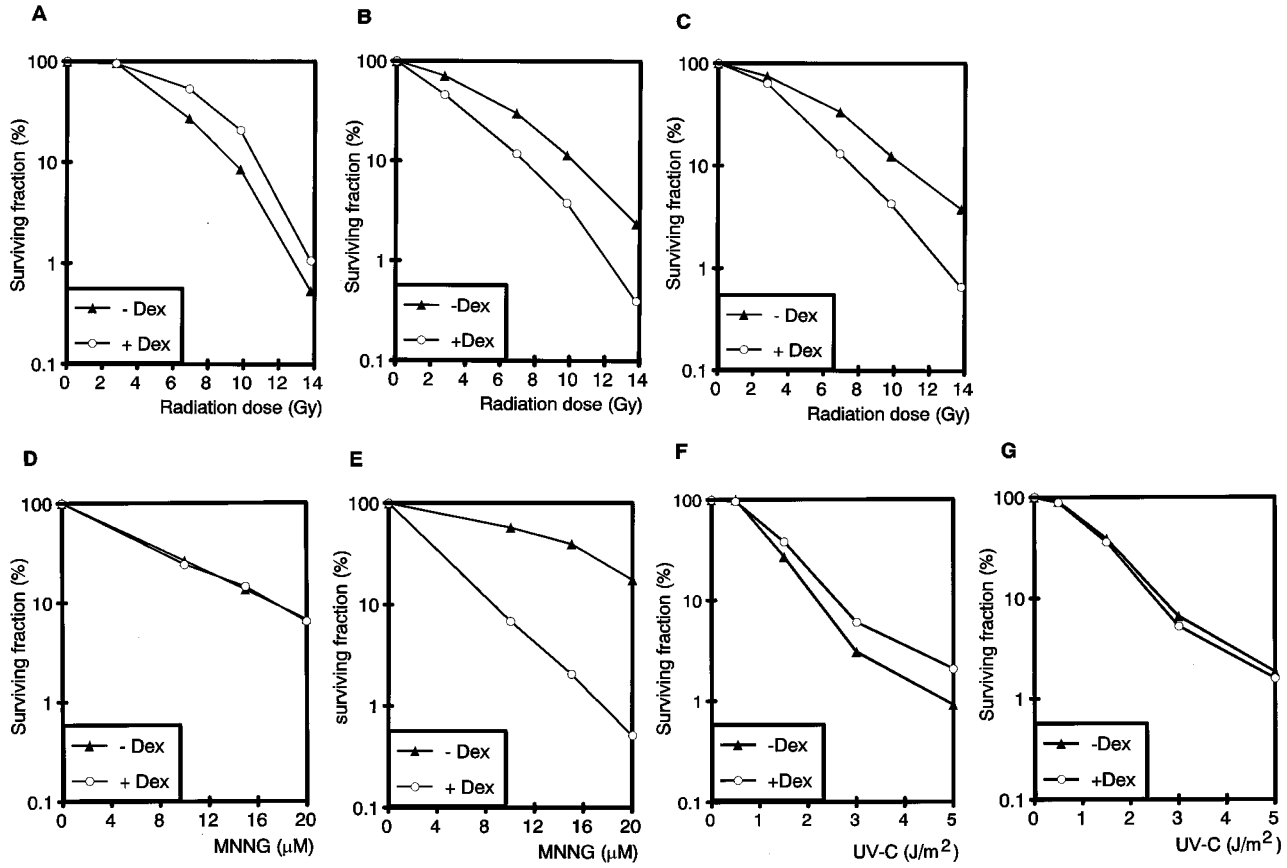

FIG. 7. Survival of COM3 cells and COR3 control cells after introduction of DNA damage. All Dex treatments were performed during the 24 h preceding genotoxic treatment. COR3 cells (A) or COM3 cells (B) were treated with 5×10^{-8} M Dex or not treated as indicated. (C) COM3 cells were treated with 10^{-8} M Dex. Cell monolayers were γ-irradiated in PBS as indicated. After further incubation for 16 h in routine medium, cells were plated in triplicate for the determination of plating efficiency. Dex (5×10^{-8} M)-treated or untreated COR3 (D) or COM3 cells (E) were plated and MNNG treated as described in Materials and Methods. Survival was assessed in quadruplicate. Dex (5×10^{-8} M)-treated or untreated COR3 (F) or COM3 cells (G) were UV-C irradiated as indicated and subsequently processed in quadruplicate for the determination of plating efficiency. For experimental details, see Materials and Methods. Standard deviations were smaller than indicated by symbol size.

agents (23). An important difference between *trans*-dominant inhibition and antisense RNA expression is that the latter cannot discriminate between the proposed dual function of PARP in cells, i.e., poly(ADP-ribosyl)ation and the binding to DNA strand breaks, whereas *trans*-dominant inhibition abrogates only the catalytic function of PARP, analogous to the mechanism of competitive PARP inhibitors.

Having studied the *trans*-dominant PARP inhibition at the biochemical level, we next investigated the biological consequences of DBD overexpression. Since PARP has been implicated in a great variety of processes such as cell proliferation, DNA replication, base excision repair, DNA amplification, cell differentiation, and aging (for reviews, see references 4, 10, 13, and 20), we began with a relatively simple biological endpoint, cell proliferation. Here we provide evidence that in our system PARP activity is not rate limiting for normal cell proliferation, even when inhibited by about 90%. First, when we induced the COM3 or COM4 transfectants with Dex to express the DBD, we observed a slight growth retardation (Fig. 5) that was due to a nonspecific effect of Dex, because the same Dex-induced growth retardation was also observed in the matched glucocorticoid receptor control cell line COR3 and to a greater extent in COR2, expressing higher levels of the receptor (Fig. 2 and 5). A second argument that PARP activity is not rate limiting for normal cell proliferation can be made from data obtained with the COC1 and COC2 cell lines. These transfectants constitutively overexpressed the PARP DBD, with consequent *trans*-dominant inhibition of poly(ADP-ribosyl)ation, but cell division rates were the same as for the parental CO60 cell line (data not shown).

We next directly addressed the question of whether DBD overexpression had an influence on DNA replication. Since viral replicons have been extensively used to study eukaryotic DNA replication, we used as a test system plasmid pYZ289, which replicates episomally in mouse cells by virtue of its mouse polyomavirus replicon, as shown previously (56). Here we show that this plasmid can replicate as an episome also in COM3 hamster cells (Fig. 6). Treatment of these cells with Dex had no effect on the accumulation of replicated plasmid. Whether *trans*-dominant PARP inhibition influences the fidelity of DNA replication remains to be investigated.

Published data on a role for poly(ADP-ribosyl)ation in DNA replication are conflicting. Lönn and coworkers observed an accumulation of unligated 10-kb replication intermediates in cells treated with the chemical PARP inhibitor 3-aminobenzamide and proposed a role for PARP activity in stimulating the efficient ligation of those intermediates into high-molecular-weight DNA (48–50). Treatment of sea urchin embryos with 3-aminobenzamide was shown to inhibit poly(ADP-ribosyl)ation of cleavage-stage histone variants and S-phase progres-

sion (31, 32). The authors proposed a role for PARP in signaling S-phase progression during sea urchin development. On the other hand, many laboratories found that in the absence of DNA damage, treatment of cell lines with 3-aminobenzamide at rather low concentrations that nevertheless should block PARP did not influence cell proliferation rates. Furthermore, although PARP was previously found to act as an elongation inhibitor of SV40 DNA replication in vitro, it was subsequently shown by the same group (25, 47) that this effect could be reversed when their monopolymerase system was complemented to a dipolymerase system by adding multimeric activator protein I (also called A1 and RF-C), the proliferating cell nuclear antigen, and DNA polymerase δ. In vitro SV40 DNA replication in crude cell extracts was not affected by 3-aminobenzamide either (25). Independently, a dipolymerase system was reconstituted for lagging- and leading-strand synthesis at the SV40 DNA replicon, consisting of eight replication factors but lacking PARP (73). Our result from living cells indicating that poly(ADP-ribose) formation is not rate limiting for polyomavirus DNA replication is in agreement with published results from in vitro SV40 DNA replication systems in which PARP was not an essential component.

To study any influence of PARP activity on the cellular response following DNA damage, we investigated the survival of COM3 cells after treatment with different DNA-damaging agents. As can be concluded from Fig. 7B and C, COM3 cells overexpressing the DBD exhibited a substantial degree of radiosensitization. In comparison with uninduced COM3 cells, the survival curve of Dex-induced cells is characterized by a smaller shoulder and a steeper slope. Apparently, the sensitizing effect of Fig. 7B and C is an underestimate, taking into account the protective effect of Dex treatment per se, as revealed in the COR3 cells (Fig. 7A). A possible influence of poly(ADP-ribosyl)ation on DNA repair and survival of γ-irradiated cells has been studied extensively in the past by using inhibitors, with conflicting results (reviewed in reference 9). Many of the previous studies were carried out with inhibitor concentrations ranging from 5 to 10 mM. Since benzamide compounds at concentrations above 1 to 2 mM were reported to cause side effects on glucose and DNA precursor metabolism (29, 53, 54, 57) and also can inhibit mono-ADP-ribosyltransferases (62), those results are difficult to interpret.

Apart from γ-irradiation, we investigated the influence of Dex induction on the survival of cells treated with the alkylating agent MNNG. Interestingly, there was no effect of Dex on the survival of COR3 cells in this case (Fig. 7D). But again, Dex-induced COM3 cells exhibited a drastic sensitization against MNNG (Fig. 7E). This result is in agreement with data from other groups showing increased sensitivity against alkylating agents in cell culture systems depleted of PARP, as obtained by antisense RNA expression (23) or by random mutagenesis of cells and selection of clones with decreased PARP protein levels (18, 77).

Our data are in line with recent data revealing an involvement of poly(ADP-ribose) accumulation in DNA base excision repair. By using a microinjection assay, it was shown that overexpression of the DBD blocks unscheduled DNA synthesis following treatment with the alkylating agent MNNG, a carcinogen known to trigger base excision repair (55). Furthermore, data from two independent in vitro systems showed that DNA repair or associated events were facilitated by poly(ADP-ribosyl)ation, especially PARP automodification, although the molecular mechanisms proposed were different (3, 65, 67). In the present study, we observe a fundamental difference between the effect of DBD overexpression on cell survival following γ-irradiation and MNNG on the one hand and UV-C on the other hand. With γ-irradiation and MNNG, both of which stimulate base excision repair, there was a substantial degree of sensitization mediated by the DBD. By contrast, in the case of UV-C, the DBD could only balance out a protective effect of Dex. This finding is fully compatible with recent data obtained in an in vitro DNA repair system, showing that poly(ADP-ribosyl)ation does influence the base excision repair of pyrimidine hydrates, which are the minor UV-C-induced DNA lesions. By contrast, the nucleotide excision repair of the major UV-C-induced lesions (e.g., pyrimidine dimers) was not affected (67). Taken together, our results show that overexpression of the DBD in COM3 cells does not disturb the cellular recovery from DNA damage in general but that it interferes with the biological response to damages stimulating base excision repair rather than nucleotide excision repair.

In conclusion, the *trans*-dominant inhibition approach allowed us to discriminate between a putative involvement of poly(ADP-ribosyl)ation in DNA replication and DNA repair, since DBD overexpression did not limit episomal DNA replication, but it did block unscheduled DNA synthesis following alkylation damage (55) and, as shown here, did reduce cell survival after DNA damage substantially. Studies are in progress to investigate in this genetic system the precise role of poly(ADP-ribosyl)ation in the biological cascade following DNA damage.

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ADDENDUM IN PROOF

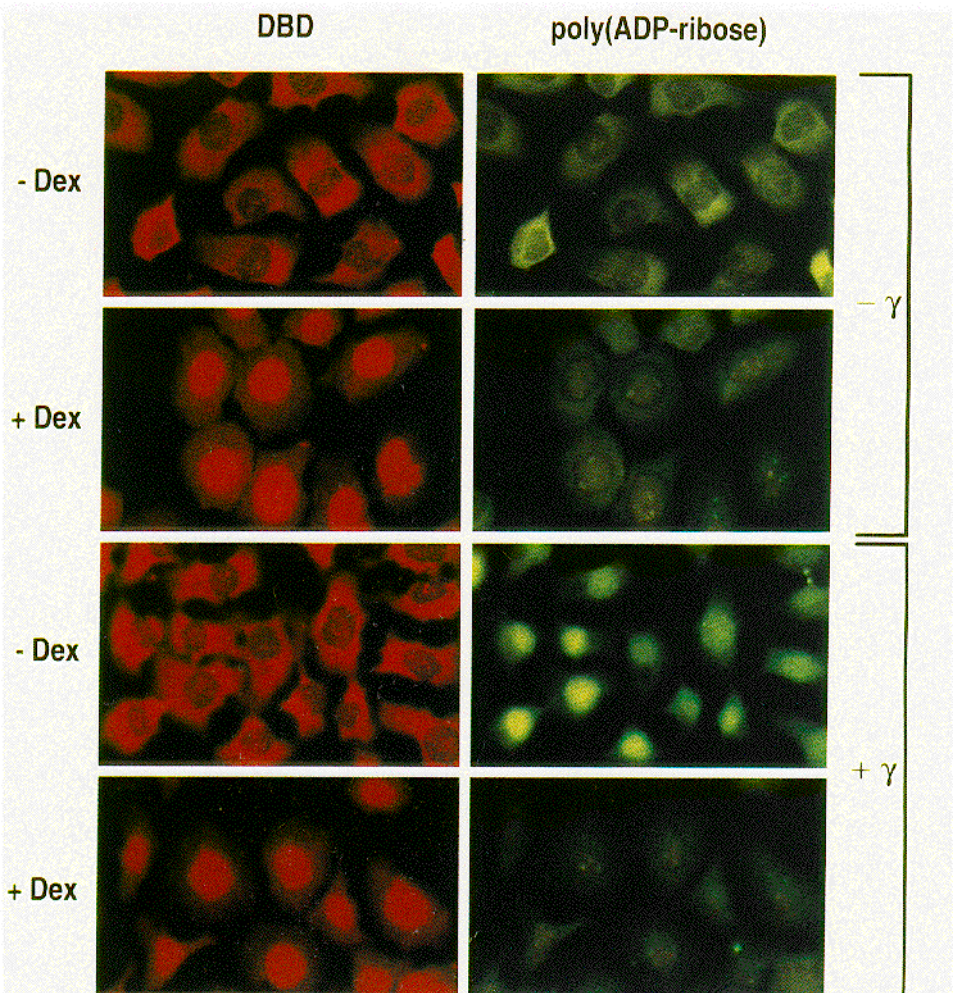
A recent report (V. Schreiber, D. Hunting, C. Trucco, B. Gowans, D. Grunwald, G. de Murcia, and J. Menissier de Murcia, Proc. Natl. Acad. Sci. USA, in press) also indicates that *trans*-dominant inhibition of poly(ADP-ribosyl)ation is associated with sensitization to MNNG.

REFERENCES

1. **Aboul-Ela, N., E. L. Jacobson, and M. K. Jacobson.** 1988. Labeling methods for the study of poly- and mono(ADP-ribose) metabolism in cultured cells. Anal. Biochem. **174:**239–250.
2. **Alksnis, M., T. Barkhem, P.-E. Ströhmstedt, H. Ahola, E. Kutoh, J.-A. Gustafsson, L. Poellinger, and S. Nilsson.** 1991. High level expression of functional full length and truncated glucocorticoid receptor in chinese hamster ovary cells. J. Biol. Chem. **266:**10078–10085.
3. **Althaus, F. R.** 1992. Poly ADP-ribosylation: a histone shuttle mechanism in DNA excision repair. J. Cell Sci. **102:**663–670.
4. **Althaus, F. R., and C. Richter.** 1987. ADP-ribosylation of proteins. Enzymology and biological significance. Mol. Biol. Biochem. Biophys. **37:**1–237.
5. **Alvarez-Gonzalez, R., and F. R. Althaus.** 1989. Poly(ADP-ribose) catabolism in mammalian cells exposed to DNA- damaging agents. Mutat. Res. **218:**67–74.
6. **Bellingham, D. L., and J. A. Cidlowski.** 1989. Stable overproduction of intact glucocorticoid receptors in mammalian cells using a selectable glucocorticoid responsive dihydrofolate reductase gene. Mol. Endocrinol. **3:**1733–1747.
7. **Berger, N. A., and S. J. Berger.** 1986. Metabolic consequences of DNA damage: the role of poly(ADP-ribose) polymerase as mediator of the suicide response. Basic Life Sci. **38:**357–363.

8. **Borek, C., W. F. Morgan, A. Ong, and J. E. Cleaver.** 1984. Inhibition of malignant transformation in vitro by inhibitors of poly(ADP-ribose) synthesis. Proc. Natl. Acad. Sci. USA **81:**243–247.
9. **Boulikas, T.** 1991. Relation between carcinogenesis, chromatin structure and poly(ADP-ribosylation) (review). Anticancer Res. **11:**489–527.
10. **Boulikas, T.** 1993. Poly(ADP-ribosyl)ation, DNA strand breaks, chromatin and cancer. Toxicol. Lett. **67:**129–150.
11. **Breathnach, R., and B. A. Harris.** 1983. Plasmids for the cloning and expression of full-length double-stranded cDNAs under control of the SV40 early or late gene promoter. Nucleic Acids Res. **11:**7119–7136.
12. **Bürkle, A., G. Chen, J. H. Küpper, K. Grube, and W. J. Zeller.** 1993. Increased poly(ADP-ribosyl)ation in intact cells by cisplatin treatment. Carcinogenesis **14:**559–561.
13. **Bürkle, A., K. Grube, and J. H. Küpper.** 1992. Poly(ADP-ribosyl)ation: its role in inducible DNA amplification, and its correlation with the longevity of mammalian species. Exp. Clin. Immunogenet. **9:**230–240.
14. **Bürkle, A., R. Heilbronn, and H. zur-Hausen.** 1990. Potentiation of carcinogen-induced methotrexate resistance and dihydrofolate reductase gene amplification by inhibitors of poly(adenosine diphosphate-ribose) polymerase. Cancer Res. **50:**5756–5760.
15. **Bürkle, A., T. Meyer, H. Hilz, and H. zur-Hausen.** 1987. Enhancement of N-methyl-N′-nitro-N-nitrosoguanidine-induced DNA amplification in a simian virus 40-transformed Chinese hamster cell line by 3-aminobenzamide. Cancer Res. **47:**3632–3636.
16. **Burnstein, K. L., D. L. Bellingham, C. M. Jewell, F. E. Powell Oliver, and J. A. Cidlowski.** 1991. Autoregulation of glucocorticoid receptor gene expression. Steroids **56:**52–58.
17. **Burnstein, K. L., C. M. Jewell, and J. A. Cidlowski.** 1990. Human glucocorticoid receptor cDNA contains sequences sufficient for receptor down-regulation. J. Biol. Chem. **265:**7284–7291.
18. **Chatterjee, S., M. F. Cheng, S. J. Berger, and N. A. Berger.** 1991. Alkylating agent hypersensitivity in poly(adenosine diphosphate-ribose) polymerase deficient cell lines. Cancer Commun. **3:**71.
19. **Cherney, B. W., O. W. McBride, D. F. Chen, H. Alkhatib, K. Bhatia, P. Hensley, and M. E. Smulson.** 1987. cDNA sequence, protein structure, and chromosomal location of the human gene for poly(ADP-ribose) polymerase. Proc. Natl. Acad. Sci. USA **84:**8370–8374.
20. **de-Murcia, G., and J. Menissier-de-Murcia.** 1994. Poly(ADP-ribose) polymerase: a molecular nick-sensor. Trends Biochem. Sci. **19:**172–176.
21. **Desmarais, Y., L. Menard, J. Lagueux, and G. G. Poirier.** 1991. Enzymological properties of poly(ADP-ribose)polymerase characterization of automodification sites and NADase activity. Biochim. Biophys. Acta **1078:**179–186.
22. **Ding, R., Y. Pommier, V. H. Kang, and M. Smulson.** 1992. Depletion of poly(ADP-ribose) polymerase by antisense RNA expression results in a delay in DNA strand break rejoining. J. Biol. Chem. **267:**12804–12812.
23. **Ding, R., and M. Smulson.** 1994. Depletion of nuclear poly(ADP-ribose) polymerase by antisense RNA expression: influences on genomic stability, chromatin organization and carcinogen cytotoxicity. Cancer Res. **54:**4627–4634.
24. **Durkacz, B. W., O. Omidiji, D. A. Gray, and S. Shall.** 1980. (ADP-ribose)n participates in DNA excision repair. Nature (London) **283:**593–596.
25. **Eki, T., and J. Hurwitz.** 1991. Influence of poly(ADP-ribose) polymerase on the enzymatic synthesis of SV40 DNA. J. Biol. Chem. **266:**3087–3100.
26. **Fasel, N., K. Pearson, E. Buetti, and H. Diggelmann.** 1982. The region of mouse mammary tumor virus DNA containing the long terminal repeat includes a long coding sequence and signals for hormonally regulated transcription. EMBO J. **1:**3–7.
27. **Giguère, V., S. M. Hollenberg, M. G. Rosenfeld, and R. M. Evans.** 1986. Functional domains of the human glucocorticoid receptor. Cell **46:**645–652.
28. **Hahn, P., B. Nevaldine, and W. F. Morgan.** 1990. X-ray induction of methotrexate resistance due to dhfr gene amplification. Somatic Cell Mol. Genet. **16:**413–423.
29. **Hunting, D. J., B. J. Gowans, and J. F. Henderson.** 1985. Specificity of inhibitors of poly(ADP-ribose) synthesis. Effects on nucleotide metabolism in cultured cells. Mol. Pharmacol. **28:**200–206.
30. **Huppi, K., K. Bhatia, D. Siwarski, D. Klinman, B. Cherney, and M. Smulson.** 1989. Sequence and organization of the mouse poly (ADP-ribose) polymerase gene. Nucleic Acids Res. **17:**3387–3401.
31. **Imschenetzky, M., M. Montecino, and M. Puchi.** 1991. Poly(ADP-ribosylation) of atypical CS histone variants is required for the progression of S phase in early embryos of sea urchins. J. Cell Biochem. **46:**234–241.
32. **Imschenetzky, M., M. Montecino, and M. Puchi.** 1993. Temporally different poly(adenosine diphosphate-ribosylation) signals are required for DNA replication and cell division in early embryos of sea urchins. J. Cell Biochem. **51:**198–205.
33. **Jacobson, E. L., and M. K. Jacobson.** 1976. Pyridine nucleotide levels as a function of normal and transformed 3T3 cells. Arch. Biochem. Biophys. **175:**627–634.
34. **Jacobson, E. L., R. Meadows, and J. Measel.** 1985. Cell cycle perturbations following DNA damage in the presence of ADP-ribosylation inhibitors. Carcinogenesis **6:**711–714.
35. **Jacobson, E. L., J. Y. Smith, K. Wielckens, H. Hilz, and M. K. Jacobson.** 1985. Cellular recovery of dividing and confluent C3H10T1/2 cells from N-methyl-N′-nitro-N-nitrosoguanidine in the presence of ADP-ribosylation inhibitors. Carcinogenesis **6:**715–718.
36. **Juarez-Salinas, H., J. L. Sims, and M. K. Jacobson.** 1979. Poly(ADP-ribose) levels in carcinogen-treated cells. Nature (London) **282:**740–741.
37. **Kasid, U. N., D. F. Stefanik, R. A. Lubet, A. Dritschilo, and M. E. Smulson.** 1986. Relationship between DNA strand breaks and inhibition of poly (ADP-ribosylation): enhancement of carcinogen-induced transformation. Carcinogenesis **7:**327–330.
38. **Kawamitsu, H., H. Hoshino, H. Okada, M. Miwa, H. Momoi, and T. Sugimura.** 1984. Monoclonal antibodies to poly(adenosine diphosphate ribose) recognize different structures. Biochemistry **23:**3771–3777.
39. **Klehr, D., K. Maass, and J. Bode.** 1991. Scaffold-attached regions from the human interferon beta domain can be used to enhance the stable expression of genes under the control of various promoters. Biochemistry **30:**1264–1270.
40. **Ko, M. S., and T. Takano.** 1989. A highly inducible system of gene expression by positive feedback production of glucocorticoid receptors. DNA **8:**127–133.
41. **Krupitza, G., and P. Cerutti.** 1989. Poly(ADP-ribosylation) of histones in intact human keratinocytes. Biochemistry **28:**4054–4060.
42. **Krupitza, G., and P. Cerutti.** 1989. ADP-ribosylation of ADPR-transferase and topoisomerase I in intact mouse epidermal cells JB6. Biochemistry **28:**2034–2040.
43. **Küpper, J. H., G. de-Murcia, and A. Bürkle.** 1990. Inhibition of poly(ADP-ribosyl)ation by overexpressing the poly(ADP-ribose) polymerase DNA-binding domain in mammalian cells. J. Biol. Chem. **265:**18721–18724.
44. **Kurosaki, T., H. Ushiro, Y. Mitsuuchi, S. Suzuki, M. Matsuda, Y. Matsuda, N. Katunuma, K. Kangawa, H. Matsuo, T. Hirose, S. Inayama, and Y. Shizuta.** 1987. Primary structure of human poly(ADP-ribose) synthetase as deduced from cDNA sequence. J. Biol. Chem. **262:**15990–15997. (Additions and corrections, **263:**7437, 1988.)
45. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
46. **Lavi, S.** 1981. Carcinogen-mediated amplification of viral DNA sequences in simian virus-40 transformed Chinese hamster embryo cells. Proc. Natl. Acad. Sci. USA **78:**6144–6148.
47. **Lee, S.-H., A. D. Kwong, Y. Ishimi, and J. Hurwitz.** 1989. Studies on the DNA elongation inhibitor and its proliferating cell nuclear antigen-dependent control in simian virus 40 DNA replication in vitro. Proc. Natl. Acad. Sci. USA **86:**4877–4881.
48. **Lönn, U., and S. Lönn.** 1985. Accumulation of 10-kilobase DNA replication intermediates in cells treated with 3-aminobenzamide. Proc. Natl. Acad. Sci. USA **82:**104–108.
49. **Lönn, U., and S. Lönn.** 1988. Step-wise progression of mammalian 10-kb DNA replication intermediates to mature chromatin. Eur. J. Biochem. **178:**47–51.
50. **Lönn, U., S. Lönn, U. Nylen, and G. Winblad.** 1989. Altered formation of DNA replication intermediates in human 46 BR fibroblast cells hypersensitive to 3-aminobenzamide. Carcinogenesis **10:**981–985.
51. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
52. **Mendoza-Alvarez, H., and R. Alvarez-Gonzalez.** 1993. Poly(ADP-ribose) polymerase is a catalytic dimer and the automodification reaction is intermolecular. J. Biol. Chem. **268:**22575–22580.
53. **Milam, K. M., and J. E. Cleaver.** 1984. Inhibitors of poly(adenosine diphosphate-ribose) synthesis: effect on other metabolic processes. Science **223:**589–591.
54. **Milam, K. M., G. H. Thomas, and J. E. Cleaver.** 1986. Disturbances in DNA precursor metabolism associated with exposure to an inhibitor of poly(ADP-ribose) synthetase. Exp. Cell Res. **165:**260–268.
55. **Molinete, M., W. Vermeulen, A. Bürkle, J. Menissier-de-Murcia, J. H. Küpper, J. H. Hoeijmakers, and G. de-Murcia.** 1993. Overproduction of the poly(ADP-ribose) polymerase DNA-binding domain blocks alkylation-induced DNA repair synthesis in mammalian cells. EMBO J. **12:**2109–2117.
56. **Moriwaki, S., T. Yagi, C. Nishigori, S. Imamura, and H. Takebe.** 1991. Analysis of N-methyl-N-nitrosourea-induced mutations of a shuttle vector plasmid propagated in O6-methylguanine-DNA methyltransferase-deficient cells in comparison with proficient cells. Cancer Res. **51:**6219–6223.
57. **Moses, K., A. L. Harris, and B. W. Durkacz.** 1988. Synergistic enhancement of 6-thioguanine cytotoxicity by ADP-ribosyltransferase inhibitors. Cancer Res. **48:**5650–5654.
58. **Okret, S., L. Poellinger, Y. Dong, and J. A. Gustafsson.** 1986. Down-regulation of glucocorticoid receptor mRNA by glucocorticoid hormones and recognition by the receptor of a specific binding sequence within a receptor cDNA clone. Proc. Natl. Acad. Sci. USA **83:**5899–5903.
59. **Ozawa, Y., K. Uchida, M. Uchida, Y. Ami, S. Kushida, N. Okada, and M. Miwa.** 1993. Isolation of cDNAs encoding the catalytic domain of poly(ADP-ribose) polymerase from Xenopus laevis and cherry salmon using heterologous oligonucleotide consensus sequences. Biochem. Biophys. Res. Commun. **193:**119–125.

60. **Phi Van, L., J. P. von Kries, W. Ostertag, and W. H. Stratling.** 1990. The chicken lysozyme 5′ matrix attachment region increases transcription from a heterologous promoter in heterologous cells and dampens position effects on the expression of transfected genes. Mol. Cell. Biol. **10:**2302–2307.
61. **Radons, J., B. Heller, A. Bürkle, B. Hartmann, M. L. Rodriguez, K. D. Kroncke, V. Burkart, and H. Kolb.** 1994. Nitric oxide toxicity in islet cells involves poly(ADP-ribose) polymerase activation and concomitant NAD+ depletion. Biochem. Biophys. Res. Commun. **199:**1270–1277.
62. **Rankin, P. W., E. L. Jacobson, R. C. Benjamin, J. Moss, and M. K. Jacobson.** 1989. Quantitative studies of inhibitors of ADP-ribosylation in vitro and in vivo. J. Biol. Chem. **264:**4312–4317.
63. **Rosewicz, S., A. R. McDonald, B. A. Maddux, I. D. Goldfine, R. L. Miesfeld, and C. D. Logsdon.** 1988. Mechanism of glucocorticoid receptor down-regulation by glucocorticoids. J. Biol. Chem. **263:**2581–2584.
64. **Saito, I., K. Hatakeyama, T. Kido, H. Ohkubo, S. Nakanishi, and K. Ueda.** 1990. Cloning of a full-length cDNA encoding bovine thymus poly(ADP-ribose) synthetase: evolutionarily conserved segments and their potential functions. Gene **90:**249–254.
65. **Satoh, M. S., and T. Lindahl.** 1992. Role of poly(ADP-ribose) formation in DNA repair. Nature (London) **356:**356–368.
66. **Satoh, M. S., and T. Lindahl.** 1994. Enzymatic repair of oxidative DNA damage. Cancer Res. **54:**1899–1901.
67. **Satoh, M. S., G. G. Poirier, and T. Lindahl.** 1993. NAD(+)-dependent repair of damaged DNA by human cell extracts. J. Biol. Chem. **268:**5480–5487.
68. **Scovassi, A. I., C. Mariani, M. Negroni, C. Negri, and U. Bertazzoni.** 1993. ADP-ribosylation of nonhistone proteins in HeLa cells: modification of DNA topoisomerase II. Exp. Cell Res. **206:**177–181.
69. **Simonin, F., J. P. Briand, S. Muller, and G. de-Murcia.** 1991. Detection of poly(ADP ribose) polymerase in crude extracts by activity-blot. Anal. Biochem. **195:**226–231.
70. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
71. **Takahashi, S., D. Nakae, Y. Yokose, Y. Emi, A. Denda, S. Mikami, T. Ohnishi, and Y. Konishi.** 1984. Enhancement of DEN initiation of liver carcinogenesis by inhibitors of NAD+ ADP ribosyl transferase in rats. Carcinogenesis **5:**901–906.
72. **Thibodeau, J., G. Gradwohl, C. Dumas, S. Clairoux-Moreau, G. Brunet, C. Penning, G. G. Poirier, and P. Moreau.** 1989. Cloning of rodent cDNA coding the poly(ADP-ribose) polymerase catalytic domain and analysis of mRNA levels during the cell cycle. Biochem. Cell Biol. **67:**653–660.
73. **Tsurimoto, T., T. Melendy, and B. Stillman.** 1990. Sequential initiation of lagging and leading strand synthesis by two different polymerase complexes at the SV40 DNA replication origin. Nature (London) **346:**534–539.
74. **Uchida, K., S. Hanai, K. Ishikawa, Y. Ozawa, M. Uchida, T. Sugimura, and M. Miwa.** 1993. Cloning of cDNA encoding Drosophila poly(ADP-ribose) polymerase: leucine zipper in the auto-modification domain. Proc. Natl. Acad. Sci. USA **90:**3481–3485.
75. **Uchida, K., T. Morita, T. Sato, T. Ogura, R. Yamashita, S. Noguchi, H. Suzuki, H. Nyunoya, M. Miwa, and T. Sugimura.** 1987. Nucleotide sequence of a full-length cDNA for human fibroblast poly(ADP-ribose) polymerase. Biochem. Biophys. Res. Commun. **148:**617–622.
76. **Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin.** 1979. DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. Proc. Natl. Acad. Sci. USA **76:**1373–1376.
77. **Witmer, M. V., N. Aboul-Ela, M. K. Jacobson, and T. D. Stamato.** 1994. Increased sensitivity to DNA-alkylating agents in CHO mutants with decreased poly(ADP-ribose) polymerase activity. Mutat. Res. **314:**249–260.
78. **Zhang, J., V. L. Dawson, T. M. Dawson, and S. H. Snyder.** 1994. Nitric oxide activation of poly(ADP-ribose) synthetase in neurotoxicity. Science **263:**687–689.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