

Overproduction of the poly(ADP-ribose) polymerase DNA-binding domain blocks alkylation-induced DNA repair synthesis in mammalian cells

Miguel Molinete¹, Wim Vermeulen², Alexander Bürkle³, Josiane Ménissier-de Murcia¹, J.Heiner Küpper³, Jan H.J.Hoeijmakers² and Gilbert de Murcia^{1,4}

¹Institut de Biologie Moléculaire et Cellulaire du Centre National de la Recherche Scientifique associé à l'Université Louis Pasteur, UPR de Cancérogénèse et de Mutagenèse Moléculaire et Structurale, 15 rue René Descartes, F-67084 Strasbourg Cedex, France, ²Department of Cell Biology and Genetics, Erasmus University, Rotterdam, PO Box 1738, NL-3000 DR Rotterdam, The Netherlands and ³Angewandte Tumorstudiologie, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 242, D-6900 Heidelberg, Germany

⁴Corresponding author

Communicated by D.Bootsma

The zinc-finger DNA-binding domain (DBD) of poly(ADP-ribose) polymerase (PARP, EC 2.4.2.30) specifically recognizes DNA strand breaks induced by various DNA-damaging agents in eukaryotes. This, in turn, triggers the synthesis of polymers of ADP-ribose linked to nuclear proteins during DNA repair. The 46 kDa DBD of human PARP, and several derivatives thereof mutated in its first or second zinc-finger, were overproduced in *Escherichia coli*, in CV-1 monkey cells or in human fibroblasts to study their DNA-binding properties, the trans-dominant inhibition of resident PARP activity, and the consequences on DNA repair, respectively. A positive correlation was found between the *in vitro* DNA-binding capacity of the recombinant DBD polypeptides and their inhibitory effect on PARP activity stimulated by the alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). Furthermore, overproduced wild-type DBD blocked unscheduled DNA synthesis induced in living cells by MNNG treatment, but not that induced by UV irradiation. These results define a critical role for the second zinc-finger of PARP for DNA single-stranded break binding and furthermore underscore the importance for PARP to act as a critical regulatory component in the repair of DNA damage induced by alkylating agents.

Key words: base excision/immunofluorescence/microinjection/nucleotide excision repair/zinc-finger protein

Introduction

Poly(ADP-ribose) polymerase (PARP; EC 2.4.2.30) is a chromatin-associated enzyme of eukaryotic cell nuclei which, in the presence of DNA breaks, covalently attaches ADP-ribose from nicotinamide adenine dinucleotide (NAD) to various nuclear acceptor proteins and transfers further ADP-ribose units onto this initial adduct to form poly(ADP-ribose). Under conditions of extensive DNA breakage, very large amounts of branched polymers are rapidly synthesized and enzymatically degraded by poly(ADP-ribose) glyco-

hydrolase, resulting in a half-life of <1 min (Alvarez-Gonzalez and Jacobson, 1987). Although PARP binds to single- or double-stranded DNA whether it is closed circular or linear, its catalytic activity is absolutely dependent on the presence of single- or double-stranded breaks (Benjamin and Gill, 1980). At the molecular level, however, the function(s) of poly(ADP-ribose) synthesis is not clear. Poly(ADP-ribosylation) has been postulated to influence a number of chromatin functions that involve nicking and resealing of DNA strands, such as the recovery from DNA damage, cell proliferation, differentiation and genetic recombination (see for review Althaus and Richter, 1987; Shall, 1989).

Poly(ADP-ribose) polymerase is a 113 kDa multifunctional enzyme (Kameshita *et al.*, 1984). Following limited proteolysis of purified protein, three functional domains in the enzyme molecule have been identified: (i) a 46 kDa fragment which acts as a DNA-binding domain (DBD) corresponding to regions A, B and C in Figure 1A. It is located in the N-terminal region which comprises two zinc-fingers involved in DNA strand break recognition (Ménissier-de Murcia *et al.*, 1989; Gradwohl *et al.*, 1990) and a bipartite nuclear location signal (region B) (Schreiber *et al.*, 1992); (ii) a central 22 kDa polypeptide containing several glutamic acid residues which are sites of auto-poly(ADP-ribosylation) (region D); and (iii) a C-terminal fragment of 54 kDa (regions E and F), encompassing the catalytic domain, which can be cut down to 40 kDa (region F) without losing its basal catalytic activity (Simonin *et al.*, 1990; see for review de Murcia *et al.*, 1991).

It has been shown that PARP needs zinc for enzyme activity (Zahradka and Ebisuzaki, 1984) and we have determined that each enzyme molecule contains two Zn(II) ions bound to the 29 kDa N-terminal fragment (region A) that is part of the DBD (Mazen *et al.*, 1988, 1989). Disruption of the metal-binding ability of the second zinc-finger (FII) dramatically reduced target DNA-binding *in vitro*. In contrast, when the Zn(II)-binding residues of the first zinc-finger (FI) were mutated, the DNA-binding activity was only slightly affected (Gradwohl *et al.*, 1990). On the other hand, by exploiting the two properties of the 46 kDa DBD, i.e. its DNA-binding capacity and its nuclear location signal (which ensures rapid and efficient targeting to the nucleus), we demonstrated that overexpression of the human PARP-DBD in transfected cells effects a trans-dominant inhibition of the resident PARP activity (Küpper *et al.*, 1990).

The ability to interfere selectively with endogenous PARP by competition for DNA breaks through overproduction of the DBD made it possible to assess the role of the enzyme in different DNA repair pathways. To this aim a series of point mutants of the human PARP-DBD was expressed in *Escherichia coli* and first characterized with respect to their *in vitro* DNA-binding properties by South-western blotting. Subsequently, they were introduced into mammalian cells, by transfection of expression constructs, to establish their *in vivo* DNA-binding and PARP-inhibiting properties. The

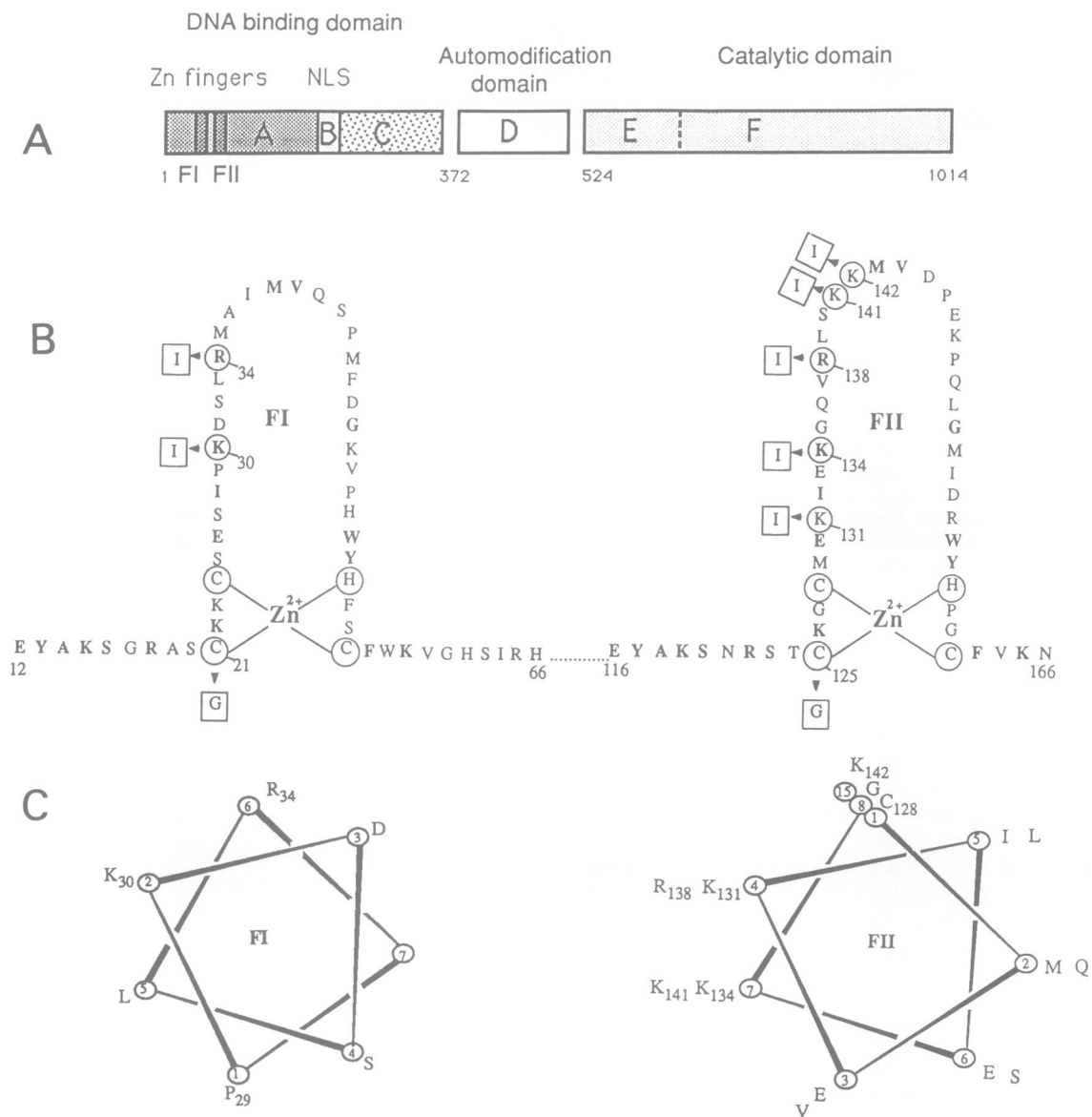


Fig. 1. Schematic representation of PARP and the zinc-fingers. (A) Representation of the three different functional domains of poly(ADP-ribose) polymerase obtained after mild trypsin and papain digestion, according to Kameshita *et al.* (1984). The first box comprises the DNA-binding domain with the zinc-fingers (region A), the nuclear location signal (NLS, region B) and region C. The second box comprises the automodification² domain (region D) and the third box the catalytic domain (regions E and F). (B) The primary structure of the two zinc-fingers is shown. Amino acid substitutions are indicated by arrowheads pointing to the mutant amino acids. (C) Helical-wheel representation of the putative α -helices of the FI and FII zinc-fingers.

effect on base excision repair (BER) and on nucleotide excision repair (NER) was investigated by microinjection of the mutant and wild-type DBD protein or DNA constructs into human fibroblasts and determination of the consequences on BER synthesis [induced by the alkylating agent *N*'-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG)] or on NER synthesis (induced by UV irradiation). We found that the trans-dominant inhibition of resident PARP, depending upon the integrity of the second zinc-finger, specifically blocks DNA repair induced by alkylation damage but not that induced by UV irradiation.

Results

In vitro DNA-binding properties of recombinant PARP-DBD

The second zinc-finger of PARP (FII) contains a short stretch of amino acids (residues Cys128–Asp145) which is

predicted to be in an α -helical configuration (Chou and Fasman, 1974) (Figure 1B). This region contains five basic residues that are potential candidates for DNA interaction. Among them, K131, K134, R138 and K141 are predicted to be on the same face of the putative α -helix (Figure 1C). In view of the preponderant role played by FII in the binding of the enzyme to nicked DNA (Gradwohl *et al.*, 1990; Ikejima *et al.*, 1990), we have systematically mutated these four basic amino acids into Ile; since FI and FII share sequence homology (Uchida *et al.*, 1987), the conserved residues in FI (Lys30 and Arg34) were also mutated into Ile (see Figure 1B). We have subcloned the cDNA encoding the DBD into the prokaryotic expression vector pTG161, overproduced the mutated proteins in *E. coli* and tested for their DNA-binding activities by South-western blotting. A ³²P-end-labelled 66 bp DNA probe harbouring a single nick at position 33 (Ménissier-de Murcia *et al.*, 1989; Gradwohl *et al.*, 1990) was used for this assay. None of the point

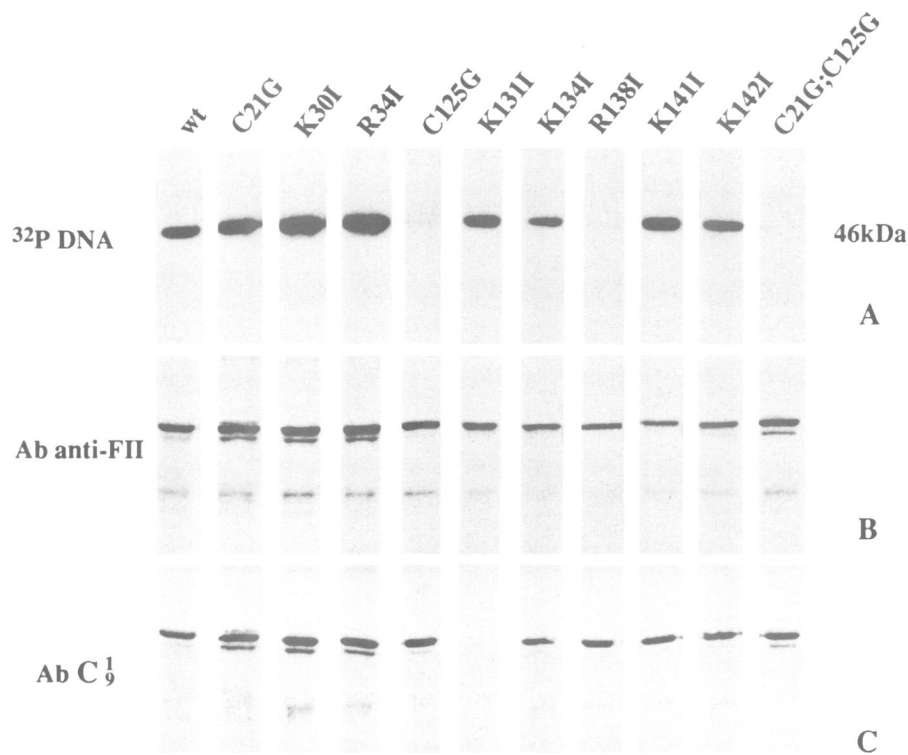


Fig. 2. DNA-binding capacity of the 46 kDa wild-type and mutant DBD polypeptides overproduced in *E. coli*. (A) South-western blotting using a 66 bp ^{32}P -end-labelled probe harbouring a nick at position 33 (Ménissier-de Murcia *et al.*, 1988). (B) Western blot analysis using an anti-FII polyclonal antibody (Simonin *et al.*, 1989). (C) Western blot analysis using the monoclonal antibody C_9 (Lamarre *et al.*, 1988).

mutations in FI significantly reduced the DNA-binding capacity of the corresponding polypeptide when compared with the wild-type DBD (Figure 2A). This was observed for the C21G mutation affecting one of the Cys residues that coordinates zinc in FI and for the two mutations affecting the basic residues of FI (K30I and R34I). In contrast, some of the FII mutations dramatically decreased the DNA-binding of the corresponding proteins, in particular the C125G mutation, which alters Cys125 (involved in zinc coordination) and R138I (altering one of the basic residues in the putative α -helix). Likewise, the double mutant C21G;C125G did not show any DNA-binding activity. In summary, our results strongly suggest that FII is involved in the interaction with DNA single-stranded breaks, and that Arg138 is crucial for this function.

The recombinant proteins were identified by Western blot experiments using a polyclonal antibody raised against a synthetic peptide corresponding to FII (Simonin *et al.*, 1991) (Figure 2B) as well as monoclonal antibody C_9 (Figure 2C), in order to verify that similar amounts of protein were loaded in each experiment. Obviously, the failure of C125G, R138I and the double mutant C21G;C125G polypeptides to bind DNA is not due to lower quantities of the corresponding proteins.

Mutation K131I appeared to abolish binding of monoclonal antibody C_9 to the DBD (Figure 2C), indicating that the amino acid K131 is closely related to the epitope recognized by this antibody.

Trans-dominant inhibition of resident PARP activity by overproduction of wild-type or mutant PARP-DBD in CV-1 cells

In order to study the DNA-binding properties of DBD mutants in living cells, we have subcloned the cDNA

encoding the wild-type PARP-DBD and different versions mutated in FI (C21G) or FII (C125G and R138I) into the eukaryotic expression vector pECV23 (Belt *et al.*, 1989). These constructs were transfected into CV-1 cells, resulting in a high level of transient expression. We exploited the trans-dominant inhibition of cellular PARP activity in transfected cells overexpressing the PARP-DBD (Küpper *et al.*, 1990) which was apparently mediated by a competition for DNA breaks necessary for the activation of the resident enzyme. The inhibition could be detected in transient transfection assays in which both DBD overproduction and poly(ADP-ribose) formation stimulated by carcinogen treatment were visualized in the same cells by double immunofluorescence. In this study, the pECV series of DBD expression constructs was transfected, and cells were challenged with the alkylating agent MNNG and assessed for trans-dominant PARP inhibition, using a slightly modified immunofluorescence protocol. Repair of MNNG-induced alkylation damage, by the action of DNA glycosylases and apurinic/aprimidinic endonucleases, results in the generation of a large number of single-stranded breaks within minutes after exposure of the cells (Lijinsky, 1976). Overproduction of the wild-type or mutant PARP-DBDs (Figure 3A–D) in CV-1 cells is clearly visualized by a strong, nuclear, red fluorescence, whereas in non-transfected neighbouring cells the resident PARP is barely detectable under these conditions. Non-transfected cells can be identified by their non-specific faint cytoplasmic background staining. The lower panel (Figure 3E–I) shows that poly(ADP-ribose) immunofluorescence staining (green) is visible only after cells have been treated with MNNG (50 μM) which induces DNA strand breaks and thus activates PARP. As expected, overexpression of the construct encoding the wild-type DBD (Figure 3F) inhibited resident

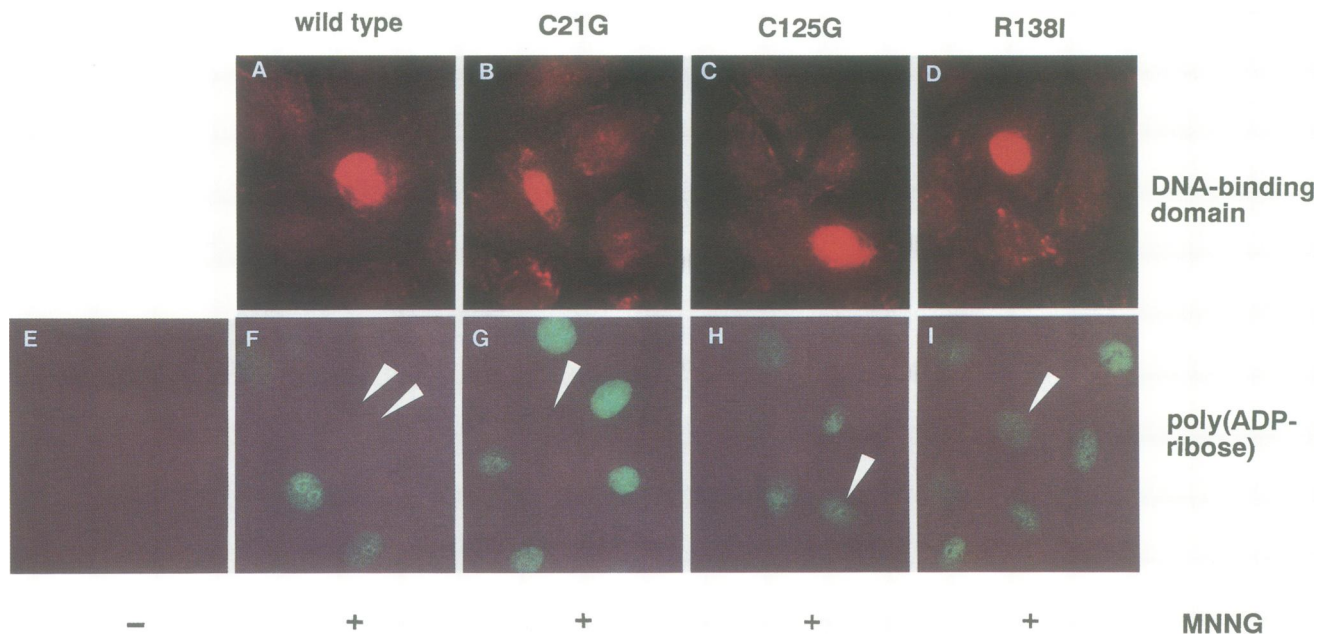


Fig. 3. Effect of the overexpression of wild-type or mutant PARP-DBD on poly(ADP-ribose) formation by resident PARP. CV-1 cells were electrotransfected with wild-type (A and F) or mutant PARP-DBD expression constructs (C21G, B and G; C125G, C and H; and R138I, D and I) and 44 h later treated with MNNG or not as indicated. Double immunofluorescence was used to detect both the overexpressed protein and poly(ADP-ribose) in the same cells. As a negative control for poly(ADP-ribose) staining, cells (transfected with R138I mutant plasmid) were immunostained without prior MNNG treatment (E). Photographic exposure times were identical for pictures of the same type of fluorescence. White arrowheads mark the positions of cells overexpressing the respective DBD. The phenotypes of the overexpressing cells shown are representative for the respective version of DBD.

PARP activity completely (within the detection limits of this assay). Complete inhibition was also observed with the mutant affecting FI (C21G; Figure 3G). In contrast, both mutants affecting FII (C125G and R138I; Figure 3H and I) failed to show any inhibition when compared with the neighbouring, non-expressing cells. The double mutant (C21G;C125G) also failed to show any inhibitory effect in this assay (data not shown). Taken together, these results demonstrate that the dominant-negative phenotype of CV-1 cells caused by overproducing the PARP-DBD perfectly matches the *in vitro* DNA strand break-binding capacity of the respective DBD mutant.

Trans-dominant inhibition of resident PARP activity blocks alkylation-induced DNA repair synthesis

The PARP-DBD was overproduced in *E. coli* and purified, in order to evaluate possible consequences of the inhibition of resident PARP activity on DNA repair after its microinjection into cultured human fibroblasts (de Jonge *et al.*, 1983). The PARP-DBD was highly overproduced after heat induction of bacteria, the recombinant protein represented ~20% of the total protein content and migrated at the predicted mol wt. in SDS-polyacrylamide gels (Figure 4, lane b). The recombinant DBD was present in the soluble fraction and was easily recovered in the clear lysate (Figure 4, lane c). Protamine sulfate precipitation was used to remove DNA, followed by ammonium sulfate precipitation (Figure 4, lanes d and e, respectively) to reduce the volume before loading the sample on a hydroxylapatite column. After this purification step the protein was almost pure (Figure 4, lane f) and subsequent single-stranded DNA cellulose column chromatography was used to remove minor contaminants. The final purified material consisted of one major band of 46 kDa as detected on a Coomassie-stained

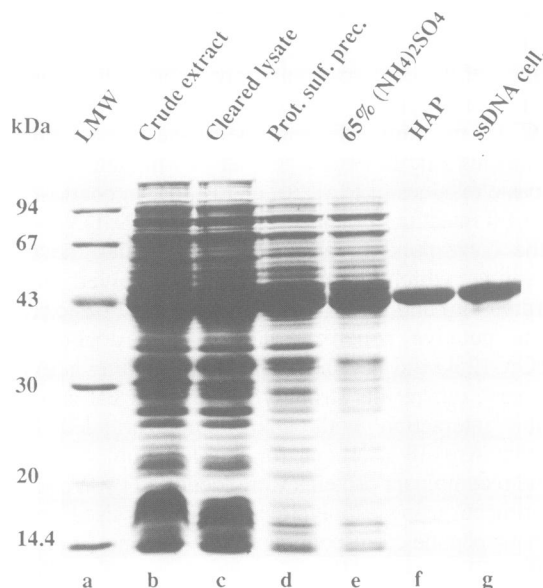


Fig. 4. Purification of the 46 kDa DBD of human poly(ADP-ribose) polymerase. Samples at various stages of the purification process were separated on a 0.1% SDS 12% polyacrylamide gel. LMW, molecular mass markers (lane a); crude extract (lane b); cleared lysate (lane c); protamine sulfate precipitation (lane d); ammonium sulfate precipitation (lane e); hydroxylapatite adsorption chromatography (lane f); single-stranded DNA cellulose affinity chromatography (lane g).

polyacrylamide gel (Figure 4, lane g). About 5 mg of PARP-DBD per litre of bacterial culture were purified using this procedure. The recombinant PARP-DBD was functional as judged by its DNA-binding properties measured by the gel retardation assay (data not shown), by its efficient nuclear translocation and by its PARP-inhibiting properties (see below).

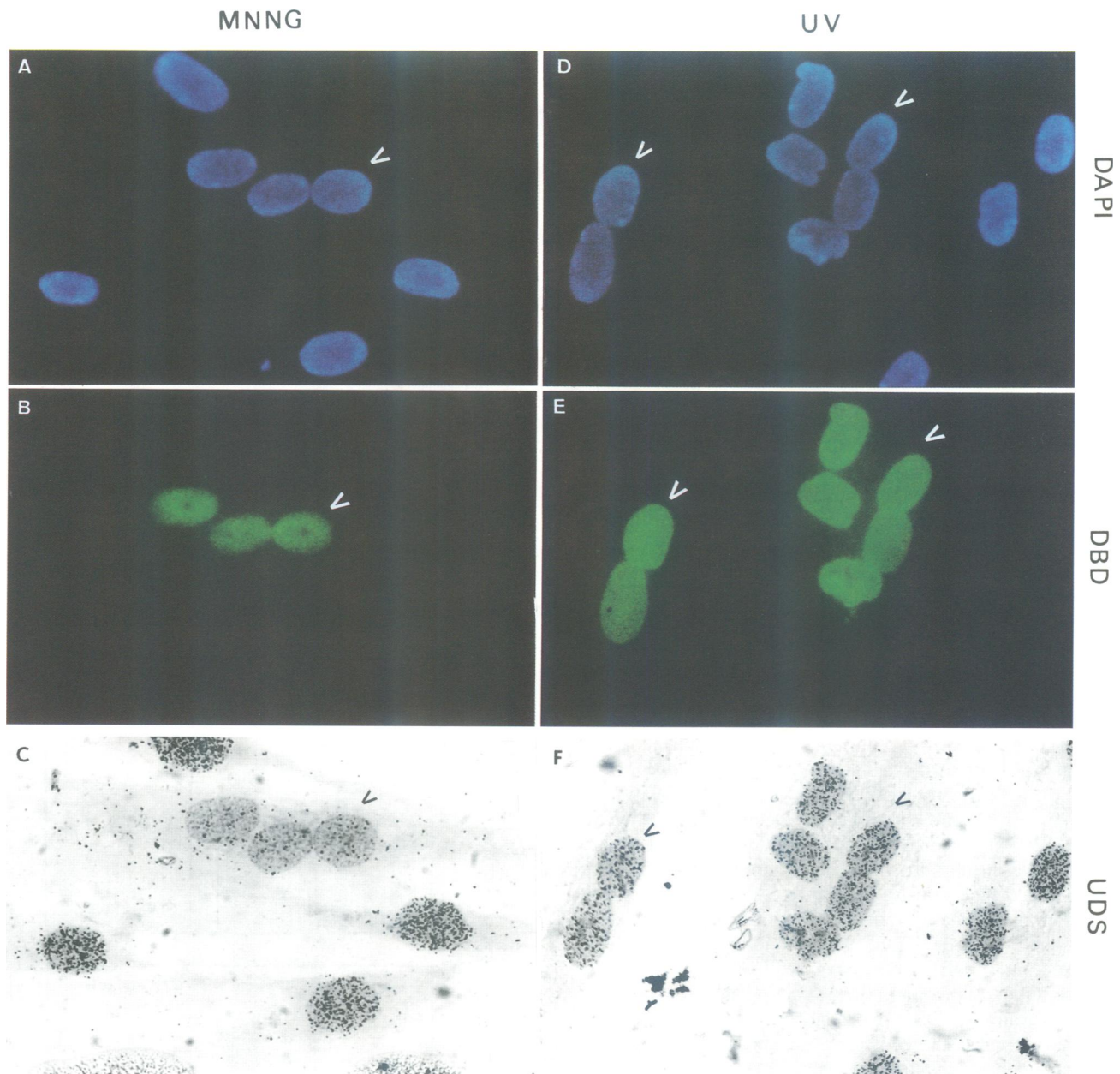


Fig. 5. Microinjection of the 46 kDa PARP-DBD protein into C5RO repair-proficient fibroblasts. Nuclei were visualized by staining with the non-intercalating DNA dye DAPI (panels A and D). Panels C and F show a micrograph of C5RO fibroblast homo-polykaryons microinjected with wild-type DBD. UDS was assayed following MNNG treatment (100 μ M; panel C) or UV irradiation (10 J/m²; panel F). Panels B and E show the corresponding fields prepared for immunofluorescence with the monoclonal C₉ anti-DBD antibody. The arrows point to the injected homo-polykaryons.

To investigate the involvement of PARP in BER and in NER, the purified PARP-DBD was introduced by microinjection into the cytoplasm of repair-proficient homo-polykaryons generated by fusion of normal (C5RO) human fibroblasts as described by de Jonge *et al.* (1983). Cells were subsequently subjected either to treatment with MNNG (100 μ M), inducing methylation damage which is mainly removed by BER, or to UV irradiation (10 J/m²) which causes formation of photoproducts which are then eliminated by NER. To visualize and quantify repair activity, the cells were—after the genotoxic treatment—assayed for unscheduled DNA synthesis (UDS) by incubation in the presence of [³H]TdR (for specific conditions see Materials and methods), followed by fixation and processing for autoradiography to detect newly synthesized DNA repair

patches. To assess DBD overproduction and UDS in the same cell, we developed a procedure that combines immunofluorescence and autoradiography (see Materials and methods). The injected cells exhibited clearly detectable quantities of the DBD polypeptide in their nuclei as demonstrated by immunofluorescence (Figure 5B and E). Furthermore, we verified that the introduced DBD efficiently inhibited cellular MNNG-induced PARP activity in the injected human fibroblasts, by double immunofluorescence for DBD and poly(ADP-ribose) performed on a separate slide injected and treated in parallel (data not shown). Figure 5 panels C and F demonstrate that both the treatment with MNNG and UV induced significant levels of UDS. To examine whether the UDS seen after MNNG treatment indeed was derived from BER (short patch repair) and was

not caused by spurious lesions eliminated by NER (long patch repair), XP-A cells (which are totally deficient in NER) were subjected to MNNG treatment and similarly assayed for UDS. The number of grains above XP-A and normal fibroblasts was similar (data not shown), thus excluding the possibility that the MNNG-induced repair synthesis was in fact derived from NER. Strikingly, the nuclei of injected homo-polykaryons displayed dramatically decreased levels of MNNG-induced UDS when compared with non-injected, neighbouring monokaryons (Figure 5C). In contrast, the UDS derived from UV lesion removal was not significantly affected by the block of PARP activity induced by the injection of the PARP-DBD (Figure 5F). UV irradiation was reported to induce poly(ADP-ribose) formation in living cells, as detected by boronate chromatography of cell extracts and quantification of retained polymers (Jacobson *et al.*, 1983). We have, however, been unable to detect polymer signals by immunofluorescence in CV-1 or human fibroblasts following UV irradiation of 0.5–500 or 2–20 J/m², respectively. This apparent discrepancy might be due to different cell culture conditions and/or the different poly(ADP-ribose) detection method. In any event, relatively low levels of UV-induced poly(ADP-ribose) formation are consistent with the lack of UDS inhibition by overproduced DBD.

To establish the specificity of the inhibition effect of the PARP-DBD on MNNG-induced UDS and to find out whether the inhibition effect was dependent on the DNA-binding properties of the PARP-DBD, mammalian expression vectors encoding the various mutated DBD polypeptides were injected intranuclearly into one of the nuclei of C5RO homo-polykaryons. After an incubation of 24 h to permit expression of the injected DNA, the effect on MNNG-induced BER was analysed. The data are presented as histograms shown in Figure 6. Upon injection of the wild-type construct (Figure 6A) two distinct cell populations can be discerned: (i) one displaying a clearly decreased level of UDS, corresponding with properly injected and DBD-expressing cells as assessed by immunofluorescence, and (ii) a subpopulation with normal UDS in which no DBD expression could be demonstrated probably because the cells were not (properly) injected or did not express the injected gene for other reasons. In contrast, when the double mutant (C21G;C125G) that does not bind DNA was overproduced, no inhibition of UDS was recorded (Figure 6D). Conversely, the corresponding single mutants displayed an intermediate behaviour, with the FI mutant (C21G) being a more potent inhibitor of UDS than the FII mutant (C125G; Figure 6B and C, respectively).

These results show that the wild-type PARP-DBD, either as a purified polypeptide or overproduced from an expression vector, strongly inhibits UDS induced by an alkylating agent but not by UV irradiation. This blocking property of the DBD is completely lost if both zinc-fingers are mutated, demonstrating the absolute requirement of DNA-break binding for the UDS inhibition to occur; however, either the first or the second zinc-finger alone is sufficient to cause partial UDS inhibition.

Discussion

Mutations affecting the second zinc-finger dramatically reduce binding to nicks in DNA

α -Helices are frequently found as recognition elements in the helix-turn-helix motifs of a number of prokaryotic and

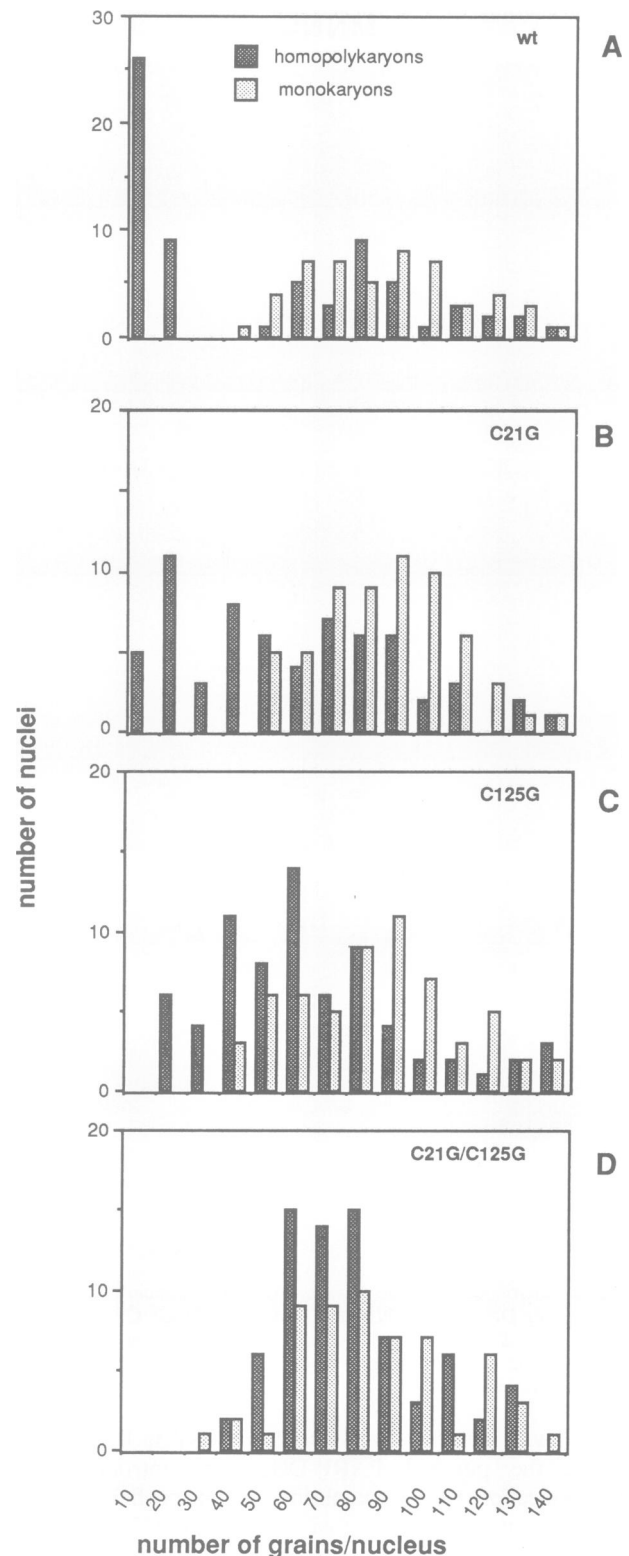


Fig. 6. Effect of the injection of the 46 kDa and mutant DBD cDNA expression plasmids on MNNG-induced UDS in C5RO primary fibroblasts. In each experiment more than 60 nuclei of homo-polykaryons were injected, assayed for their number of grains per nucleus and scored accordingly (dark columns). Likewise, 50 monokaryons (non-S-phase) were evaluated as an internal standard and positive control (light columns). Histograms representing the scores of the wild-type DBD cDNA (A), FI C21G mutant cDNA (B), FII C125G mutant cDNA (C) and double mutant cDNA (D) microinjections are shown. UDS inhibition by overproduced DBD is evidenced by the appearance of cells with low grain-per-nucleus counts.

eukaryotic genetic regulatory proteins (Luisi, 1992). This is also true of the three structural classes of zinc-fingers crystallized so far: the Zif 268 protein which belongs to the TFIIIA zinc-finger family (Pavletich and Pabo, 1991), the glucocorticoid receptor (Luisi *et al.*, 1991) and the Gal4 protein (Marmorstein *et al.*, 1992). They have in common, within their DBD, an α -helix which lies in the major groove of the DNA and directs interactions with the bases of the target sequence. In PARP, a putative α -helix in the second zinc-finger might be a candidate for the interaction with single strand breaks in DNA. The five basic residues present in FII, as well as the corresponding basic amino acids of FI, were mutated into Ile. All mutated DBD polypeptides display the same DNA-binding capacity as wild-type, with the exception of three, all of which were altered in the second zinc-finger: C125G, R138I and C21G;C125G. Furthermore, the epitope corresponding to the monoclonal antibody C₃ was found to be associated with the Lys131 region, adjacent to the critical residue Arg138. This antibody is the only one that has been reported to inhibit *in vitro* PARP activity substantially (Lamarre *et al.*, 1988). It is interesting to note that the two basic residues Lys131 and Arg138, which appear to be important for DNA-binding and/or activity, have been conserved during evolution and are located on the same face of the predicted α -helix two turns apart. Although these results do not prove that such an α -helix exists, they indicate that FII, and especially the amino acid Arg138, are crucial for binding of the 46 kDa DBD to nicks, confirming the results described by Gradwohl *et al.* (1990) in the context of a 29 kDa PARP N-terminal fragment.

Trans-dominant inhibition of nick-induced PARP activity requires the second zinc-finger

The effects on the trans-dominant inhibition of the cellular resident PARP of point mutations affecting FI (C21G) or FII (C125G, R138I) or both (C21G;C125G) were examined in eukaryotic cells overexpressing the wild-type or mutated versions of the DBD cDNA in the pECV vector. In perfect agreement with the *in vitro* DNA-binding results shown in Figure 2A, we found a trans-dominant inhibition of the endogenous PARP activity not only in cells overproducing the wild-type DBD, as expected (Küpper *et al.*, 1990), but also in FI-mutated versions. In contrast, the FII-mutant DBD which did not bind DNA *in vitro* failed to inhibit the resident PARP activity. Thus, point mutations in crucial parts of FII abolish the trans-dominant inhibition of cellular PARP. The most plausible interpretation is that the impaired DNA-binding of the mutated DBD polypeptide no longer permits competition with the endogenous enzyme for single-stranded breaks. Consistent with this notion is the observation that the inhibition is also abrogated by an excess of single-stranded breaks (Küpper *et al.*, 1990). Taken together, our results show that in living cells carrying single-stranded DNA breaks, the trans-dominant phenotype is related to the integrity of FII and that the underlying mechanism is a competition for DNA strand breaks which are a necessary cofactor for activating the enzyme.

PARP interferes with DNA repair induced by alkylating agents

The monofunctional alkylating agent MNNG induces a diverse set of methylation damage including N^3 -methyl adenine, and N^3 - and N^7 -methyl guanine as the main

products, and O^6 -methyl guanine as the major mutagenic lesion. A large fraction of these lesions is removed by base excision repair, involving the action of specific DNA glycosylases that catalyse the excision of free bases, thereby generating abasic sites in the DNA. These are, in turn, the substrate for apurinic/apyrimidinic endonucleases which induce single-stranded breaks, followed by limited exonuclease action, resynthesis and ligation. In contrast, UV-induced photoproducts (mainly cyclobutane pyrimidine dimers and 6–4 photoproducts) are recognized and eliminated by a principally different mode of repair: the nucleotide excision pathway. This repair process consists globally of five steps carried out in a complex reaction mechanism by the concerted action of multiple proteins: (i) lesion recognition, (ii) dual incision in the damaged strand at some distance around the lesion (Huang *et al.*, 1992), (iii) excision of the oligonucleotide containing the injury, (iv) gap-filling DNA synthesis and (v) ligation. The first three steps are probably performed by one or more multienzyme complexes [see Friedberg (1985) for a comprehensive review on DNA repair]. In our microinjection experiments, the PARP-DBD, either as injected protein or as the product of an injected expression construct, induced a drastic reduction of BER-associated UDS. In contrast with MNNG inducing UDS, the microinjection of the wild-type DBD had no significant effect on UDS induced by UV exposure. These results indicate that, *in vivo*, PARP is indeed targeted to sites of DNA strand breaks induced during the repair of alkylation-type lesions, and that the DBD interferes with rather early steps, such as excision and/or polymerization. That the DBD does not inhibit UV-induced UDS is probably related to the apparently low levels of stimulation of cellular PARP activity by UV damage in both CV-1 cells and human fibroblasts. This could be due to the low steady-state level of breaks in DNA following UV treatment of the cells (James and Lehmann, 1982; Cleaver *et al.*, 1983) or to the masking of the repair patch by the human excinuclease (Huang *et al.*, 1992).

Satoh and Lindahl (1992) have recently shown in an essentially histone-free *in vitro* DNA repair system that NAD-starved (i.e. inactive) PARP present in the cell extract inhibited DNA repair, as compared with the active enzyme. Interestingly, depletion of PARP from the crude extract had the same effect as had providing NAD for enzyme catalysis. This suggests that the nick-binding activity of PARP, residing in its DBD, may block free DNA ends against attack by the repair enzymes, and that this block can be relieved by allowing PARP to become catalytically active and auto-modified. Our data on the effect of the 46 kDa DBD on repair synthesis, obtained in living cells, are fully consistent with those *in vitro* results, since the introduction of an excess of catalytically inactive DBD molecules led to inhibition of UDS, taken as a measure of *in vivo* repair (Figure 6).

On the other hand, our data are also entirely consistent with an alternative mechanistic model to explain the function of poly(ADP-ribosyl)ation at the molecular level, i.e. the relaxation or the opening of chromatin superstructure by covalent or non-covalent modification of histones, thus allowing access for the DNA repair machinery (de Murcia *et al.*, 1988; Althaus, 1992). Inasmuch as the PARP-DBD acts as a trans-dominant inhibitor of poly(ADP-ribosyl)ation, of course it would not allow other DNA repair factors to access the damaged site.

Whatever the molecular mechanism of action of

poly(ADP-ribosyl)ation ultimately turns out to be, the striking ability of the PARP-DBD to block MNNG-induced nicks and thus to prevent DNA repair synthesis highlights the potential for PARP to act as a critical regulatory component in DNA repair of living cells.

Materials and methods

Cell lines, culture conditions and cell fusion

CV-1 cells were grown in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY) containing 10% fetal calf serum and 1% penicillin/streptomycin. Cells were maintained in a humidified incubator with a 5% CO₂ atmosphere.

C5RO primary human fibroblasts were cultured in Ham's F10 medium supplemented with 11% fetal calf serum and penicillin/streptomycin. Homopolykaryons to be injected were generated by fusion of cells using inactivated Sendai virus, as described by de Weerd-Kastelein *et al.* (1972). The fused cell population was seeded onto 0.6 cm × 0.8 cm areas of a microscope slide provided with a 2 mm grid and cultured for at least 3 days prior to microinjection.

Construction of PARP-DBD expression vectors

A stop codon at position 1119 and a *Pst*I site at position 1124 in the PARP cDNA were introduced by PCR. The resulting PCR product was cloned into the *Pst*I site of the *E. coli* expression vector pTG161 (Transgène; Strasbourg) resulting in plasmid pTG 46k, or in the *Xho*I site of the eukaryotic expression vector pECV23 (Belt *et al.*, 1989) using *Pst*I–*Xho*I linkers, resulting in pECV 46k.

Site-directed mutagenesis

The 1124 bp *Pst*I cDNA fragment encoding the PARP-DBD was purified from pTG 46k and cloned into the polylinker site of phage vector M13mp18. Oligonucleotides (15–20mers) were used in Amersham's oligonucleotide-directed *in vitro* mutagenesis system under the conditions described by the supplier. After mutagenesis the cDNAs were sequenced and cloned back into the *Pst*I site of pTG161 or into the *Xho*I site of pECV23.

Analysis of bacterially expressed proteins

E. coli TGE 900 cells (Transgène, Strasbourg) transformed with recombinant plasmids were grown at 28°C to an absorbance at 600 nm of 0.8 in LB medium with 100 µg ampicillin/ml, and then heat-induced at 37°C for 5 h as described by Courtney *et al.* (1984). Crude extracts were prepared as described by Gradwohl *et al.* (1990). The bacterial protein samples were electrophoresed in SDS–12% polyacrylamide minigels and electrotransferred onto nitrocellulose sheets (BAS 83, Schleicher & Schuell) according to Towbin *et al.* (1979) at 4°C in a miniblott apparatus for 1 h at 200 mA.

For DNA-binding assays, the blots were treated according to the protocol of Ménessier-de Murcia *et al.* (1989) with a 66 bp, ³²P-end-labelled DNA probe (10⁷ c.p.m./µg) harbouring a nick at position 33.

Immunoreactive polypeptides were revealed as described by Mazen *et al.* (1989).

Purification of the 46 kDa DNA-binding domain

Cultures of plasmid-containing TGE 900 cells were grown and heat-induced as described by Courtney *et al.* (1984). The culture was centrifuged for 10 min at 7000 g. All subsequent steps were carried out at 4°C. The cells were homogenized in buffer A (50 mM Tris–HCl pH 8, 1 mM EDTA, 10% glycerol, 100 mM NaCl, 10 mM 2-mercaptoethanol; 0.1 mM PMSF) at 10 ml/2 g cells. Tween 20 (0.2%) and NP40 (0.2%) were added to the solution and stirred for 30 min. The cell lysate was cleared by centrifugation at 20 000 g for 10 min. Protamine sulfate (Sigma, St Louis, USA) was added to the cleared lysate at a final concentration of 5 mg/ml, and precipitated DNA and proteins were pelleted by centrifugation at 20 000 g for 10 min. Solid ammonium sulfate (Merck, Darmstadt, Germany) was then slowly added to the supernatant to reach 65% saturation, stirred for 1 h and centrifuged at 20 000 g for 10 min. The supernatant was discarded and the precipitated material was resuspended in buffer B (2 mM K₂HPO₄, 1 mM KH₂PO₄, 10% glycerol, 10 mM 2-mercaptoethanol, 0.1 mM PMSF pH 7.3). This sample was loaded on to a hydroxylapatite column (HA Ultrogel, IBF) washed with buffer B, and eluted with a linear gradient of 3–500 mM phosphate in the same buffer. The elution fractions containing the polypeptide were then dialysed against buffer A and concentrated on a Centricon apparatus using a YM10 filter (Amicon Division, W.R. Grace & Co.-conn., Beverly, USA). The sample was then loaded on a single-stranded DNA cellulose column (Sigma), washed with buffer A and eluted with a linear gradient of 0.1 M to 1.2 M NaCl in the same buffer. The

elution fractions containing the protein were pooled, dialysed and concentrated on a Centricon apparatus in 50 mM Tris–HCl pH 8, 20% glycerol. A sample of each step was stored for further analysis on SDS gel and quantification of protein by the absorbance method. The final concentration of the 46 kDa protein was ~6 mg/ml.

Transfection of CV-1 cells and immunocytochemistry

The eukaryotic expression vector pECV encoding the 46 kDa wild-type or mutated human PARP-DBD was digested with *Cl*aI and *S*aII and purified using a GeneClean II kit (BIO 101, La Jolla, CA). Five micrograms of each of these DNAs, each combined with 15 µg pBluescript (Stratagene) as carrier, were transfected into CV-1 monkey cells by electroporation exactly as described by Küpper *et al.* (1990). Cells were plated on coverslips and, after 44 h, treated with 50 µM MNNG (Serva, Heidelberg, Germany) for 15 min at 37°C. Coverslips were rinsed twice with PBS and fixed with ice-cold 10% trichloroacetic acid, 2% tetrasodium pyrophosphate for 10 min, followed by washing in 70%, 90% and absolute ethanol for 3 min each at –20°C. Indirect double immunofluorescence was performed as described by Küpper *et al.* (1990), with some modifications. Briefly, cells fixed on coverslips were successively incubated with the following antibodies: (i) a rabbit antiserum against the second zinc-finger of PARP (Simonin *et al.*, 1991; diluted 1:100 in 1% BSA in PBS); (ii) tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit immunoglobulins (Nordic, Tilburg, The Netherlands; diluted 1:100); (iii) a crude hybridoma cell culture supernatant containing mouse monoclonal antibody 10H against poly(ADP-ribose) (Kawamitsu *et al.*, 1984); and (iv) fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulins (Bioyeda, Rehovot, Israel; diluted 1:50). Incubations were carried out in a humid chamber at 37°C for 45 min (for primary antibodies) or for 30 min, respectively. Following each antibody incubation coverslips were washed 3 × 5 min in PBS. Photomicrographs were taken using a Leitz Dialux 22 EB microscope.

Microinjection and UDS experiments

Purified 46 kDa protein (2 µg/ml) was microinjected with a glass microneedle into the cytoplasm of fused human fibroblasts (C5RO cells). Thirty minutes later, the cells were exposed to UV light (10 J/m²; 254 nm) or MNNG (final concentration 100 µM) and incubated with [³H]TdR (60 µCi/ml, specific activity 40–100 Ci/mmol) for 3 h. To increase the sensitivity of the UDS assay some modifications to the standard procedure were introduced as described by Vermeulen *et al.* (1986). The cells were then fixed with 100% cold ethanol for 10 min at –20°C. Immunofluorescence was carried out as described above. Cells were incubated with monoclonal antibody C₃ against the 46 kDa protein (primary antibody), then with a FITC-conjugated goat anti-mouse immunoglobulin (secondary antibody). The cells were covered with a photographic emulsion (Kodak NTB2), exposed for 3 days, developed and stained with Giemsa solution. The pECV expression plasmids (DNA concentration 0.5 µg/µl) were injected into one of the nuclei of homo-polykaryons. Cells were incubated for 24 h to permit expression of the injected DNA and treated in the same way as after the protein injections.

In each experiment more than 60 homo-polykaryons and monokaryons (non-S-phase) were evaluated for their thymidine incorporation. The data obtained are displayed as histograms, each bar representing the number of nuclei with a given grain-per-nucleus count.

Acknowledgements

We wish to thank Dr T.Herning for help with the DBD purification, Dr C.Backendorf for the gift of the pECV expression vector, Dr G.Poirier for providing monoclonal antibody C₃ and Dr M.Miwa for the gift of monoclonal antibody 10H. We are indebted to Dr J.Lindsley, Prof. S.Shall and Dr N.G.J.Jaspers for helpful comments and discussions. This work was supported by the Association pour la Recherche contre le Cancer (ARC), by the Ligue Nationale Française Contre le Cancer and by the Deutsche Forschungsgemeinschaft (BU 698/2-1). M.M. was supported by predoctoral fellowships from the Association pour la Recherche contre le Cancer, Federation of European Biochemical Societies, EMBO and the Concerted Action on DNA Repair and Cancer. W.V. was supported by the Dutch Cancer Society (IKR88-2 and EUR92-118).

References

- Althaus, F.R. (1992) *J. Cell Sci.*, **102**, 663–670.
- Althaus, F.R. and Richter, C. (1987) *Mol. Biol. Biochem. Biophys.*, **37**, 1–126.

- Alvarez-Gonzalez, R. and Jacobson, M.K. (1987) *Biochemistry*, **26**, 3218–3224.
- Belt, P.B.G.M., Groeneveld, H., Teubel, W.J., van de Putte, P. and Backendorf, C. (1989) *Gene*, **4**, 407–417.
- Benjamin, R.C. and Gill, D.M. (1980) *J. Biol. Chem.*, **255**, 10502–10508.
- Chou, P.Y. and Fasman, G.D. (1974) *Biochemistry*, **13**, 222–245.
- Cleaver, J.E., Bodell, W., Morgan, W. and Zelle, B. (1983) *J. Biol. Chem.*, **10**, 9058–9068.
- Courtney, M., Buchwalder, A., Tessier, L.H., Jaye, M., Benavente, A., Balland, A., Kohli, V., Lathe, R., Tolstoshev, P. and Lecocq, J.P. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 669–673.
- de Jonge, A.J.R., Vermeulen, W., Keijzer, W., Hoeijmakers, J.H.J. and Bootsma, D. (1983) *EMBO J.*, **2**, 637–641.
- de Murcia, G., Huletsky, A. and Poirier, G.G. (1988) *Biochem. Cell Biol.*, **66**, 626–635.
- de Murcia, G., Ménissier-de Murcia, J. and Schreiber, V. (1991) *BioEssays*, **13**, 455–462.
- de Weerd-Kastelein, E.A., Keijzer, W. and Bootsma, D. (1972) *Nature*, **238**, 80–83.
- Friedberg, E.C. (1985) *DNA Repair*. Freeman and Company, San Francisco.
- Gradwohl, G., Ménissier-de Murcia, J., Molinete, M., Simonin, F., Koken, M., Hoeijmakers, J.H.J. and de Murcia, G. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 2990–2994.
- Huang, J.-C., Svoboda, D.L., Reardon, J.T. and Sancar, A. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 3664–3668.
- Ikejima, M., Noguchi, S., Yamashita, R., Ogura, T., Sugimura, T., Gill, M. and Miwa, M. (1990) *J. Biol. Chem.*, **265**, 21907–21913.
- Jacobson, E.L., Antol, K.M., Juarez-Salinas, H. and Jacobson, M.K. (1983) *J. Biol. Chem.*, **258**, 103–107.
- James, M.R. and Lehman, A.R. (1982) *Biochemistry*, **21**, 4007–4013.
- Kameshita, I., Matsuda, Z., Taniguchi, T. and Shizuta, Y. (1984) *J. Biol. Chem.*, **259**, 4770–4776.
- Kawamitsu, H., Hoshino, H., Miwa, M., Momoi, H. and Sugimura, T. (1984) *Biochemistry*, **23**, 3771–3777.
- Küpper, J.-H., de Murcia, G. and Bürkle, A. (1990) *J. Biol. Chem.*, **265**, 18721–18724.
- Lamarre, D., Talbot, B., de Murcia, G., Laplante, C., Leduc, Y., Mazen, A. and Poirier, G. (1988) *Biochim. Biophys. Acta*, **950**, 147–160.
- Lijinsky, W. (1976) *Progr. Nucleic Acid Res. Mol. Biol.*, **17**, 247–269.
- Luisi, B.F. (1992) *Nature*, **356**, 379–380.
- Luisi, B.F., Xu, W.X., Otwinowski, Z., Freedman, L.P., Yamamoto, K.R. and Sigler, P.B. (1991) *Nature*, **352**, 497–505.
- Marmorstein, R., Carey, M., Ptashne, M. and Harrison, S.C. (1992) *Nature*, **356**, 408–414.
- Mazen, A., Gradwohl, G. and de Murcia, G. (1988) *Anal. Biochem.*, **172**, 39–42.
- Mazen, A., Ménissier-de Murcia, J., Molinete, M., Simonin, F., Gradwohl, G., Poirier, G.G. and de Murcia, G. (1989) *Nucleic Acids Res.*, **17**, 4689–4698.
- Ménissier-de Murcia, J., Molinete, M., Gradwohl, G., Simonin, F. and de Murcia, G. (1989) *J. Mol. Biol.*, **210**, 229–233.
- Pavletich, N.K. and Pabo, C.O. (1991) *Science*, **252**, 809–817.
- Satoh, M.S. and Lindahl, T. (1992) *Nature*, **356**, 356–358.
- Schreiber, V., Molinete, M., Boeuf, H., de Murcia, G. and Ménissier-de Murcia, J. (1992) *EMBO J.*, **11**, 3263–3269.
- Shall, S. (1989) *Biochem. Soc. Trans.*, **17**, 317–322.
- Simonin, F., Ménissier-de Murcia, J., Poch, O., Muller, S., Gradwohl, G., Molinete, M., Penning, C., Keith, G. and de Murcia, G. (1990) *J. Biol. Chem.*, **265**, 19249–19256.
- Simonin, F., Briand, J.P., Muller, S. and de Murcia, G. (1991) *Anal. Biochem.*, **195**, 226–231.
- Towbin, H., Staehelin, T. and Gordon, I. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350–4354.
- Uchida, K., Morita, T., Sato, T., Ogura, T., Yamashita, R., Noguchi, S., Suzuki, H., Nyunoya, H., Miwa, M. and Sugimura, T. (1987) *Biochem. Biophys. Res. Commun.*, **148**, 617–622.
- Vermeulen, W., Ossewijer, P., de Jonge, A.J.R. and Hoeijmakers, J.H.J. (1986) *Mutat. Res.*, **165**, 199–206.
- Zahradka, P. and Ebisuzaki, K. (1984) *Eur. J. Biochem.*, **142**, 503–509.

Received on December 30, 1992