

The second zinc-finger domain of poly(ADP-ribose) polymerase determines specificity for single-stranded breaks in DNA

(DNA-binding protein/site-directed mutagenesis/zinc-binding protein/DNase I protection assay)

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ABSTRACT Poly(ADP-ribose) polymerase (EC 2.4.2.30) is a zinc-binding protein that specifically binds to a DNA strand break in a zinc-dependent manner. We describe here the cloning and expression in *Escherichia coli* of a cDNA fragment encoding the two putative zinc fingers (FI and FII) domain of the human poly(ADP-ribose) polymerase. Using site-directed mutagenesis, we identified the amino acids involved in metal coordination and analyzed the consequence of altering the proposed zinc-finger structures on DNA binding. Disruption of the metal binding ability of the second zinc finger, FII, dramatically reduced target DNA binding. In contrast, when the postulated Zn(II) ligands of FI were mutated, the DNA binding activity was only slightly affected. DNase I protection studies showed that the FII is involved in the specific recognition of a DNA strand break. These results demonstrate that poly(ADP-ribose) polymerase contains a type of zinc finger that differs from previously recognized classes in terms of both structure and function.

Poly(ADP-ribose) polymerase (PARP; EC 2.4.2.30) is a chromatin-associated enzyme of eukaryotic cell nuclei that catalyzes the covalent attachment of ADP-ribose units from the coenzyme NAD⁺ to various nuclear acceptor proteins (reviewed in ref. 1). PARP is a multifunctional enzyme (2) and its activity is strongly stimulated by DNA strand breaks (3). This posttranslational modification has been postulated to influence a number of chromatin functions, especially those involving nicking and rejoining of DNA strands, such as cell proliferation, differentiation, and recovery from DNA damage (reviewed in ref. 1). In this last case, very large amounts of poly(ADP-ribose) are rapidly synthesized and degraded with a half-life of <1 min (1). This rapid turnover suggests that this process could mediate rapid transient changes in chromatin structure (4). PARP needs zinc for its activity (5), and we determined recently that each molecule of enzyme contains two Zn(II) ions located in a 29-kDa N-terminal fragment included in the DNA-binding domain (6). Further, we demonstrated that zinc is essential for the binding of this fragment to DNA (6). These results are consistent with the presence of the duplicated motif (Cys-Xaa₂-Cys-Xaa₂₈₋₃₀-His-Xaa₂-Cys)₂ in the N-terminal part of the amino acid sequence of the enzyme (7–9), which may form two zinc-coordinated “fingers” based on the structural motifs described for the *Xenopus* 5S rRNA transcription factor TFIIIA (10, 11) or steroid receptors (12–14). Similar putative zinc-finger sequences have been identified in many nucleic acid-binding proteins (reviewed in ref. 15), and this structure appears to be a commonly used structural motif for DNA recognition. Unlike the majority of the zinc-finger proteins,

no specific sequence interaction has been shown for PARP. However, our recent DNase I protection studies demonstrated that PARP specifically binds to a single-stranded break in DNA by its metal-binding domain, dependent on the presence of Zn(II) (16). These results suggest that PARP zinc fingers may be involved in the recognition of DNA interruptions.

In this report, we describe the use of the DNA polymerase chain reaction (PCR) (17) to isolate a cDNA clone encoding the zinc-binding domain of human PARP. The expression of this cDNA fragment in *Escherichia coli* under the control of the P_L promoter of phage λ produced a PARP polypeptide that was functional in terms of zinc and DNA binding. Various deletion and point mutants of the zinc-finger domain were expressed in *E. coli* and tested for their zinc-binding ability. In a series of DNA binding and DNase I footprint experiments, we investigated the role of PARP zinc fingers in DNA interaction and especially in the recognition of DNA strand breaks.

MATERIALS AND METHODS

Oligodeoxynucleotides. The following were synthesized on an Applied Biosystems 380B DNA synthesizer and purified by HPLC: oligonucleotide A, 5'-AGGGCTTTTTCAGCT-TACTATCCT-3' (*Hind*III site underlined); oligonucleotide B, 5'-GGCCTGCAGGGGAGGATGCGGAGTCTTCG-3' (*Pst* I site and start codon underlined); oligonucleotide C, 5'-TTCTGCAGTTATTAAGCTTACTATCCTTGT-3' (*Pst* I site and overlapping stop codon/*Hind*III site underlined). Oligonucleotides A and C are antisense primers; B is a sense primer.

Cloning of the Zinc-Binding Domain. (See Fig. 1.) Ten micrograms of poly(A)⁺ RNA of human chronic myelogenous leukemia K-562 cells (18) in 9 μl of anneal buffer (250 mM KCl/Tris-HCl, pH 8.3 at 42°C/1 mM EDTA) was added to 1 μl of primer A (1 μg/μl) and heated at 90°C for 3 min. After transfer to 55°C for 30 min, 15 μl of cDNA buffer (24 mM Tris-HCl, pH 8.3 at 42°C/16 mM MgCl₂/0.4 mM each dNTP) and 5 units of avian myeloblastosis virus reverse transcriptase (Pharmacia) were added and the primer was extended at 42°C for 60 min. The cDNA sample was added to 55 μl of *Taq* polymerase buffer (100 mM Tris-HCl, pH 8.3 at 42°C/30 mM (NH₄)₂SO₄/9 mM MgCl₂/10 mM 2-mercaptoethanol) plus 20 μl of dNTP solution (each dNTP at 5 mM), 10 μl of dimethyl sulfoxide, 1.25 μl of 2 M KCl, 1 μl of primer A (1 μg/μl), and 1 μl of primer B (1 μg/μl). After heat denaturation (92°C for 7 min), 2 units of *Thermus aquaticus* (*Taq*) DNA polymerase (Perkin-Elmer/Cetus) was added and 30 cycles of amplification were performed (annealing at

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Abbreviations: PARP, poly(ADP-ribose) polymerase; PCR, polymerase chain reaction; FI, finger I; FII, finger II.

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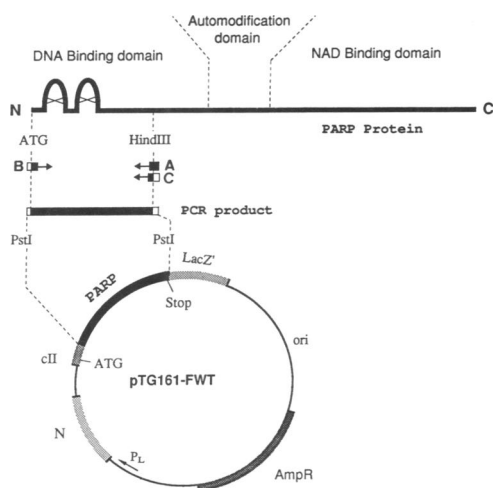


FIG. 1. Construction of the fusion protein expression vector pTG161-FWT. The cDNA fragment encoding the human PARP zinc-binding domain was mutated to introduce a stop codon at Glu-235 and *Pst* I sites at both extremities. The PARP cDNA was then cloned in the *Pst* I site of pTG161 expression vector (Transgène, Strasbourg, France; ref. 19) to form pTG161-FWT. Transcription of the cloned sequence proceeds from the major leftward promoter of bacteriophage λ (P_L), which is controlled by the host (TGE900)-encoded thermosensitive repressor cI857. After heat induction, pTG161-FWT should result in the expression of a fused polypeptide consisting of 259 amino acids: 13 derived from the N terminus of the λ cII protein fused with 12 derived from the polylinker sequence and 5' noncoding sequence of the PARP gene, and the first 234 amino acids of human PARP. Primers used for PCR are depicted as boxes with arrows. The open parts of the boxes represent the foreign sequences introduced during the amplification (*Pst* I sites and stop codon); the solid parts represent PARP coding sequence. Primers A and C are complementary to (+)-strand, B to (-)-strand.

50°C, 1 min; extension at 70°C, 5 min; denaturation at 92°C, 1 min; final extension, 5 min). The PCR product was purified by agarose gel electrophoresis and inserted between the *Pst* I and *Hind*III sites of pBS (-) plasmid (Stratagene), resulting in vector pBS-FWT.

Construction of Expression Vector. A stop codon and a *Pst* I site were introduced at the 3' end of the 714-base-pair (bp) *Pst* I–*Hind*III PARP cDNA fragment (purified from pBS-FWT) by PCR as described above. Primers B and C were used for the amplification; the annealing was done at 25°C. The resulting PCR product was cloned in the *Pst* I site of the *E. coli* expression vector pTG161 (Transgène, Strasbourg, France; ref. 19), resulting in plasmid pTG161-FWT.

Induction of Cultures. The expression constructs were used to transform *E. coli* TGE900 cells (19). Cultures of plasmid-containing TGE900 cells were grown and heat-induced as described (19). For analysis of total proteins, the cultures (5 ml) were centrifuged and bacteria were resuspended in 300 μ l of 50 mM glucose/25 mM Tris·HCl, pH 8/10 mM EDTA plus 100 μ l of sample buffer (50 mM Tris·HCl, pH 6.8/6 M urea/6% 2-mercaptoethanol/3% SDS/0.003% bromophenol blue). Cells were then lysed by sonication (one 60-sec pulse at 180 V) and the bacterial protein sample was incubated for 15 min at 65°C before SDS/PAGE analysis (20).

Site-Directed Mutagenesis. The 726-bp *Pst* I cDNA fragment encoding the PARP zinc-finger region was purified from pTG161-FWT and cloned into the polylinker site of phage vector M13. Oligonucleotides (15- to 20-mers) were used in the oligonucleotide-directed *in vitro* mutagenesis system (Amersham) under conditions described by the supplier. Substitutions are described in Table 1. After mutagenesis the cDNAs were sequenced by the dideoxynucleotide chain-termination method (21) and cloned back in the *Pst* I site of *E. coli* expression vector pTG161. The Δ KpnI mutant was constructed by deletion of the *Kpn* I internal fragment of pTG161-FWT. The G21,G125 double point mutant was obtained by replacing the *Kpn* I fragment from pTG161-G21 with that of pTG161-G125.

Analysis of Induced Proteins. Crude extracts of plasmid-containing TGE900 cells prepared as described above were electrophoresed in SDS/12% polyacrylamide minigels. After electrophoresis, the proteins were stained or were electrotransferred onto nitrocellulose membranes (BA83; 0.22- μ m pore size, Schleicher & Schuell) according to Towbin *et al.* (22) at 4°C in a miniblott apparatus for 1 hr at 200 mA. Immunoreactivity and zinc blotting experiments were performed as described (6, 16). For DNA binding and DNase I footprint assays, the blots were washed for 30 min in 50 mM Tris·HCl, pH 8/150 mM NaCl/0.1% Nonidet P-40), preincubated for 30 min at room temperature in the DNA binding buffer (20 mM Tris·HCl, pH 8/0.1 M KCl/50 μ M ZnCl₂/2 mM MgCl₂/2 mM dithiothreitol/0.1% Nonidet P-40). The blots were then incubated for 1 hr at 4°C in sealed bags with 1 ml of binding buffer containing 20 ng of a ³²P-end-labeled 66-bp DNA probe (10⁷ cpm/ μ g) harboring a strand break at position 33 (16). After 3 washes in binding buffer at 4°C, the blots either were dried and subjected to autoradiography to visualize the protein–DNA complexes or were autoradiographed wet for 1 hr so that the filter-bound protein–DNA complex could be excised and used for DNase I footprint assays as described previously by Ménessier de Murcia *et al.* (16) in a blot and footprint procedure (23).

Table 1. Zn(II)- and DNA-binding activity of fusion proteins with mutations in the PARP zinc-finger domain

Mutant	Nucleotide change(s)	Amino acid change(s)	Zn(II) binding, %	DNA binding, %
G21	T ⁶¹ to G	Cys-21 to Gly	62	68
R50	A ¹⁴⁹ to G	His-50 to Arg	97	81
R53	A ¹⁵⁸ to G	His-53 to Arg	48	75
G56	T ¹⁶⁶ to G	Cys-56 to Gly	60	81
G125	T ³⁷³ to G	Cys-125 to Gly	34	3
H162	T ⁴⁸⁴ to C, G ⁴⁸⁵ to A	Cys-162 to His	40	11
G21,G125	T ⁶¹ to G, T ³⁷³ to G	Cys-21 to Gly, Cys-125 to Gly	32	0
Δ KpnI	Δ (157–474)	Δ (53–158)	52	6

After densitometric analysis of the immunostained filter (Fig. 3) and autoradiograms (Fig. 4), the Zn(II) and DNA binding results were expressed after correction for differences in the amount of PARP polypeptide present in the samples. The results are expressed as percentages relative to the wild-type polypeptide Zn(II)- and DNA-binding activity.

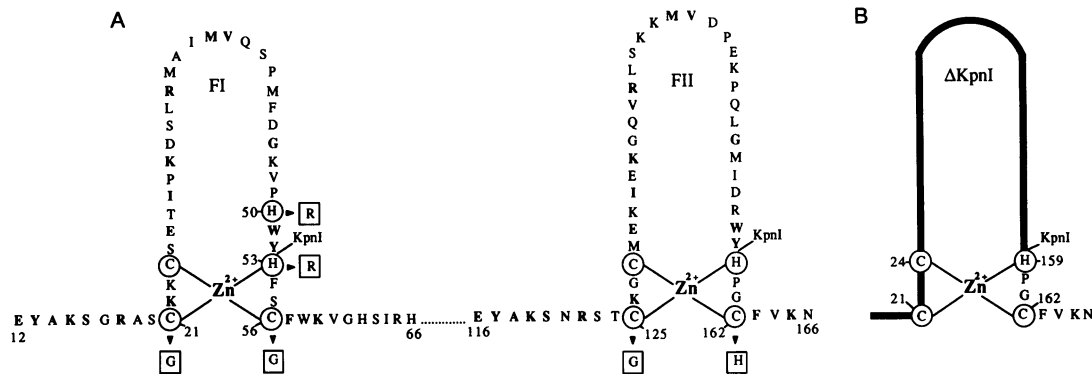


FIG. 2. Structure of the zinc-binding domain and mutagenesis. (A) The zinc-binding domain is drawn to show two zinc-coordinated fingers. Finger I (FI) is shown using Cys-21, Cys-24, His-53, and Cys-56 to coordinate Zn(II), and finger II (FII) is shown using Cys-125, Cys-128, His-159, and Cys-162 to coordinate Zn(II). Duplicated amino acids are shown in boldface according to refs. 6 and 7. Amino acid substitutions are indicated by arrowheads pointing to the mutant amino acids. Locations of *Kpn*I sites are shown next to the sequence. The mutants are described in Table 1. (B) Putative hybrid zinc finger (FI/FII) of Δ KpnI deletion mutant. Heavy line represents FI sequence.

RESULTS AND DISCUSSION

Cloning and Expression of the PARP Zinc-Binding Domain in *E. coli*. cDNA encoding the N-terminal part of human PARP containing the two putative zinc fingers was cloned by the PCR (17), using a set of oligonucleotides flanking the zinc-binding domain of the enzyme (Fig. 1). The oligonucleotide sequences were chosen on the basis of the known human cDNA sequence (7–9). The PCR amplification resulted in a single 714-bp band of specific PARP cDNA (data not shown). The PCR product was then cloned in pBS(-) vector (Stratagene). Several positive clones (pBS-FWT) were sequenced to demonstrate that the 5' cDNA for human PARP had been cloned. Only one difference with the published sequence (7–9) was found at the amino acid level, namely, Thr-27 instead of serine. To express the zinc-binding domain in *E. coli*, the 714-bp cDNA fragment (*Pst*I–*Hind*III) was mutated by PCR to introduce an in-frame codon and a *Pst*I restriction site downstream of the natural *Hind*III site. The PCR product was cloned in the *Pst*I site of the *E. coli* expression vector pTG161, resulting in plasmid pTG161-

FWT (Fig. 1). Upon temperature-shift induction, *E. coli* strain TGE900 harboring pTG161-FWT overexpressed a 35-kDa fusion protein containing the first 234 amino acids of PARP, spanning the entire zinc-binding domain. The polypeptide represented $\approx 15\%$ of total cell protein and was recognized both by polyclonal antibodies (see Fig. 3) and by a monoclonal antibody (C_b) (data not shown) specific for the DNA-binding domain (24) raised against the bovine PARP.

Zinc Binding by Mutant Fusion Proteins. To identify the amino acids implicated in the coordination of the two zinc ions, we performed a mutational analysis of the two putative zinc fingers (FI and FII) by making deletion and specific single or double amino acid exchanges of the postulated zinc ligands (Fig. 2; Table 1). Mutated cDNAs were then cloned in the pTG161 expression vector and zinc binding of the mutant fusion proteins expressed in *E. coli* was assessed by a simple Zn(II) blot assay (25). All the mutated polypeptides were overexpressed and the corresponding molecular masses were consistent with the length of the cloned cDNAs (Fig. 3A). The proteins were recognized by specific anti-PARP

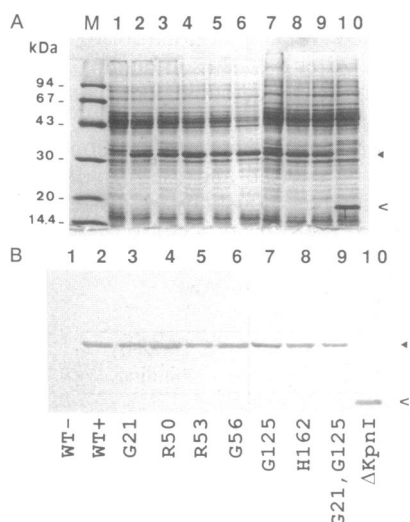


FIG. 3. Analysis of *E. coli* extracts containing wild-type or mutant PARP zinc-finger domain. Plasmid-containing TGE900 cells were grown and induced (see *Materials and Methods*), and extracts were analyzed by SDS/PAGE. Lanes: 1, pTG161-FWT noninduced; 2, pTG161-FWT induced; 3, G21; 4, R50; 5, R53; 6, G125; 7, H162; 8, G21, G125; 9, Δ KpnI; M, molecular mass markers. (A) Coomassie-stained gel. (B) Immunoblot analysis using polyclonal antibody to calf thymus PARP. Positions of the fusion proteins are indicated at right.

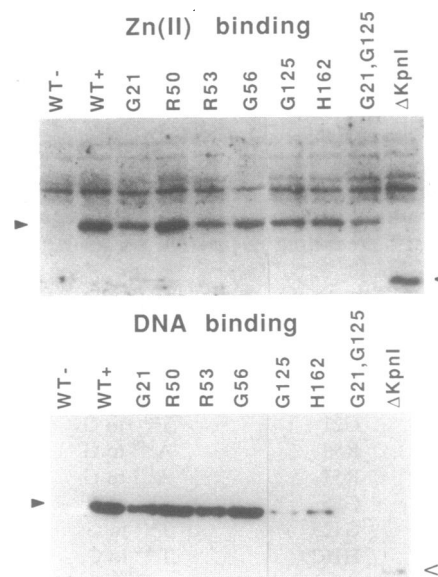


FIG. 4. Zinc and DNA binding activity. Wild-type [WT, noninduced (-) or induced (+)] and mutant PARP fusion polypeptides in crude *E. coli* extracts were subjected to SDS/12% PAGE, transferred to nitrocellulose, and probed either with $^{65}\text{Zn(II)}$ (Upper) or with a ^{32}P -end-labeled DNA probe harboring a single-strand break (Lower). The blots were autoradiographed. Arrowheads mark the position of the fusion proteins.

antibodies raised against the purified calf thymus enzyme (Fig. 3B). For the first Cys/His cluster, the most likely folding possibility is Cys-Xaa₂-Cys-Xaa₂₈-His-Xaa₂-Cys (residues 21–56) (Fig. 3); however, Cys-Xaa₂-Cys-Xaa₂₅-His-Xaa₂-His (residues 21–53) is possible too, as His-50 could also act as a zinc ligand. Mutant protein R50, in which His-50 was replaced with arginine (an amino acid that cannot bind zinc), bound as much zinc as the wild-type polypeptide (Fig. 4). This result indicates that His-50 is not involved in the formation of a putative zinc finger. In contrast, all other single point mutants in FI (G21, R53, and G56) bound 40–50% less zinc than the wild type (Table 1), suggesting that the amino acids that were replaced serve as zinc ligands in FI. This confirms the first hypothesis, in which Cys-21, Cys-23, His-53, and Cys-56 can be folded into a fingerlike structure (although Cys-23 was not tested). Still, by analogy with other finger proteins, the second Cys/His cluster is expected to form a loop between Cys-125/Cys-128 and His-159/Cys-162. Zinc binding experiments showed that mutants G125 and H162 are affected in their metal binding capacity, confirming the proposed FII structure displayed in Fig. 2. Remarkably, when Cys-162 was replaced by histidine (mutant H162), a decrease in zinc binding was observed. Since histidine substitution represents the most conserved functional change (as zinc can bind to either cysteine or histidine), this indicates that there is a stringent requirement for cysteine at position 162. Surprisingly, the G21,G125 double mutant bound zinc, even though neither the FI nor the FII structure should be possible in this protein since glycine is supposed not to permit efficient binding of metal ions such as Zn(II). Retention of zinc binding by this mutant may be due to the formation of an

alternative zinc finger, namely, His-Xaa₂-Cys-Xaa₅-His-Xaa₃-His (residues 53–66). However, this structure is unlikely to exist under normal conditions since His-62 is not conserved in the mouse PARP sequence (26). The Δ KpnI deletion mutant (Fig. 2) was constructed to provide a hybrid zinc finger between FI and FII. Interestingly, the Δ KpnI mutant was not recognized by C₅ (data not shown), a monoclonal antibody specific for the DNA-binding domain (24), probably because the corresponding epitope was located in the deleted part (residues 53–158). The resulting PARP-related polypeptide bound approximately half the amount of zinc bound by the wild-type polypeptide, suggesting that Cys-21, Cys-24, His-159, and Cys-162 can be folded into a fingerlike structure.

FII Is Essential for DNA-Binding Activity. Since DNase I protection studies had shown that PARP specifically binds to a DNA strand break by its metal-binding domain in a zinc-dependent manner (16), we asked whether PARP zinc-finger structures are involved in interaction with DNA and especially with DNA strand breaks. For that purpose bacterially expressed PARP fusion proteins mutated in the zinc ligands of FI or FII were analyzed by "Southwestern" and DNase I footprint assays (16). For Southwestern analysis, the same PARP mutant polypeptides as described above, in crude lysates, were separated electrophoretically in SDS/polyacrylamide gels and transferred to nitrocellulose. DNA-binding proteins were visualized by incubation with radiolabeled DNA and autoradiography. We used a ³²P-end-labeled 66-bp DNA probe harboring a single-strand break at position 33 (16). By comparing the amounts of radiolabeled DNA bound to similar amounts of different PARP mutant proteins, the ability of each polypeptide to bind to the nick-containing DNA probe was assessed (Table 1). Each single mutation affecting the FII motif (G125 and H162) dramatically decreased the DNA-binding capacity of the corresponding polypeptide (to 89% and 97%, respectively) when compared to the wild type (Fig. 4). In contrast, the PARP mutant proteins in which FI has been altered (G21, R53, and G56) did not show a strong decrease in DNA binding. The G21,G125 double mutant showed no DNA-binding activity. These results strongly suggest first that PARP zinc fingers are involved in DNA interaction and that FII is essential for DNA binding. Interestingly, the putative FI/FII hybrid zinc finger (Δ KpnI) binds DNA approximately to the same level as mutants affected in FII. This suggests that the residues of FII region lacking in this hybrid finger (residues upstream from His-159) might be involved in DNA interactions whereas the C-terminal amino acids of FII may not, in contrast to what was observed for steroid receptors (27–29). However, it may be that the C-terminal amino acids of FII are also involved in interactions with DNA, but together with amino acid residues in the N-terminal part.

In a second set of experiments we investigated the role of PARP fingers in the recognition of single-strand breaks in DNA. For that purpose DNase I protection experiments were performed with FI mutants, using the same DNA probe as described above. The "blot and footprint" procedure of Huet and Sentenac (23) was utilized. Bacterial lysates containing PARP polypeptides corresponding to the wild-type or mutated DNA-binding domain were fractionated by SDS/12% PAGE and the proteins were transferred to nitrocellulose. The filter was then incubated with the 5'-end-labeled probe and DNase I digestion was performed directly on the complexes bound to nitrocellulose after washing to remove unbound probe. This technique allows the analysis of bacterially expressed PARP mutant proteins directly in an *E. coli* crude extract without further purification. The footprint with PARP wild-type DNA-binding domain expressed in *E. coli* was the same as that observed with purified calf thymus PARP (Fig. 5). This result demonstrates that the fusion

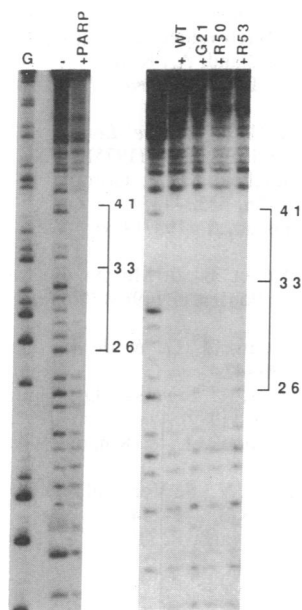


FIG. 5. DNase I footprint assays for the protection of a single-strand break in DNA by the PARP zinc-finger domain expressed in *E. coli*. Wild-type (WT) and mutant PARP polypeptides in crude *E. coli* lysates were subjected to SDS/12% PAGE, transferred to nitrocellulose, and incubated with a ³²P-end-labeled double-stranded DNA probe (66 bp) harboring a single-strand break at position 33 (16). DNase I footprint assays were performed as described under *Materials and Methods*. (Left) Lane G, degradation products of the guanine-specific sequencing reaction; lane -, control for DNA degradation without PARP; lane +PARP, footprint of purified calf thymus PARP. (Right) Lane 1, control for DNA degradation without PARP; lane +WT, footprint of PARP wild-type zinc-finger domain; lanes +G21, +R50, and +R53; footprints of G21, R50, and R53 mutants. Protected region extending 7 nucleotides on either side of position 33 is bracketed.

polypeptide is correctly folded and functional with respect to its DNA-binding capacity. R53 and G21 mutants, in which F1 was altered, showed the same protection pattern as the wild-type polypeptide. This result strongly suggests that FI is not involved in the recognition of single-strand breaks in DNA. That both the wild-type and FI-mutated polypeptides (FII remaining intact) bind to a single-strand break, and that FII is essential for DNA-binding activity, strongly suggests that FII plays a key role in the recognition of single-strand breaks.

The work presented here defines a class of DNA-binding zinc-finger domain having no sequence homology to existing classes. Both the size of the loops between the inner ligands and the DNA-binding activity differ from those of the archetypal zinc-finger class. Our results show the importance of two repeated motifs in PARP in binding to DNA interruptions. Each sequence has a four-liganded Zn(II) ion, which is essential for its integrity, and can be folded into a fingerlike structure. The data show that, in our experimental system, FII binds considerably more tightly to target DNA than does FI. Nevertheless, the results do not completely rule out a role for FI in DNA binding. One could speculate that DNA binding is stabilized by nonspecific DNA contacts contributed by region FI or that FI is involved in protein-protein contacts between two PARP molecules. The second hypothesis is consistent with the fact that when PARP binds to a single-strand break, a symmetrical footprint of 7 nucleotides on each side of the nick is observed. Indeed, both the symmetry and the size of the protected region suggest that PARP binds as a dimer, and one could imagine that FI might be involved in dimerization of DNA-binding domains. A similar situation has been postulated for steroid receptors (12). The cloning of these mutants in the complete enzyme should allow us to further clarify the role of the two zinc fingers in the stimulation of PARP activity by DNA strand breaks.

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