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Specific interaction between DNA polymerase II (PolD) and RadB, a Rad51/Dmc1 homolog, in *Pyrococcus furiosus*

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ABSTRACT

Pyrococcus furiosus **has an operon containing the DNA polymerase II (PolD) gene and three other genes. Using a two-hybrid screening to examine the interactions of the proteins encoded by the operon, we identified a specific interaction between the second subunit of PolD (DP1) and a Rad51/Dmc1 homologous protein (RadB). To ensure the specific interaction between these two proteins, each gene in the operon was expressed in *Escherichia coli* or insect cells separately and the products were purified. The *in vitro* analyses using the purified proteins also showed the interaction between DP1 and RadB. The deletion mutant analysis of DP1 revealed that a region important for binding with RadB is located in the central part of the sequence (amino acid residues 206–498). This region has an overlap to the C-terminal half (amino acids 334–613), which is highly conserved among euryarchaeal DP1s and is essential for the activity of PolD. Our results suggest that, although RadB does not noticeably affect the primer extension ability of PolD *in vitro*, PolD may utilize the RadB protein in DNA synthesis under certain conditions.**

INTRODUCTION

In living cells, the molecular machineries for DNA replication and recombination play crucial roles in the maintenance of genetic stability. In *Escherichia coli*, DNA repair synthesis is mainly processed by DNA polymerase I (PolI), which catalyzes gap-filling synthesis in addition to joining Okazaki fragments on the lagging strand during DNA replication. DNA polymerase III synthesizes DNA with high processivity on both strands during DNA replication (1). The machinery of DNA synthesis and repair is more complicated in eukaryotes than in prokaryotes. Three DNA polymerases, Pol α, Pol δ and Pol ε, are involved in eukaryotic DNA replication (reviewed in 2,3). Pol α carries a primase activity for both the leading and lagging strands. Pol δ and Pol ε extend the primers formed by Pol α. Although the functional roles of some components, including these three DNA polymerases, have been elucidated, the specific roles of Pol δ and Pol ε remain to be clarified.

Archaea, the third domain of living organisms (4), look like bacteria in terms of their cellular structure. However, their proteins involved in the genetic information system (DNA replication, repair, recombination, transcription and translation) have similar sequences to those in the Eukaryote domain. This conservation has been demonstrated clearly by the recent reports of the entire genomic sequences of some archaeal organisms (5–8). To investigate DNA metabolism in Archaea, we cloned the genes for DNA polymerase I (PolI) and II (PolII) from the hyperthermophilic archaeon, *Pyrococcus furiosus* (9,10). Unlike PolI, which contains a single peptide classified as a family B (α-like) DNA polymerase, PolII is composed of the two subunits, DP1 and DP2. The amino acid sequence of DP1 has some similarity to the second subunit of eukaryotic Pol δ, while DP2 has no similarity to any known sequence, except for the archaeal orthologs (11). PolI and PolII have been proposed to be designated PolBI and PolD, respectively, to match the polymerases and the families they belong to (12).

The genes for DP1 (*polB*) and DP2 (*polC*) of *P.furiosus* are located in tandem in an operon that encodes proteins that are thought to be related to DNA replication and recombination. One is a protein homologous to Orc1 (origin recognition complex protein 1) belonging to the cdc18^{+}/CDC6 family, and the other is similar to the Rad51/Dmc1 family proteins, including Rad55 and Rad57 (10,12,13).

A sequence analysis of the open reading frames (ORFs) in *Pyrococcus horikoshii*, which shares the same genus with *P.furiosus*, suggested that most of the genes in the same operons encode proteins with biologically related function (14). To elucidate the functional meaning of this possible replicational operon in *P.furiosus*, we investigated the interactions of its component proteins, and found a specific interaction between DP1 and the Rad51/Dmc1 homolog among the combinations of the members. The latter protein, which is now called RadB (15), seems to be related to DNA recombination. Because PolD (PolII) is suggested from its biochemical properties to be a replicational DNA polymerase (10), our present report may provide a direct linkage between replicational and recombinational molecular machineries.

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Table 1. The primers for PCRs used in this study

Target region	Direction[a]	Sequence[b]
ORF1	F	5'-CGCGGATCCATGAACGAAGGTGAACACCAGATCAAACTGGACGAGC-3'
	R	5'-GCTCGAGCTTAGATCAACCTCCTCTGCTC-3'
DP1	F	5'- GGGATCCCCAGGAATTCCCCATATGGATGAATTTGTAAAATCACTTCTGAAAGCT-3
	R	5'-GCTCGAGTCAGCACCACCCACTAAAGTCCAAAACC-3'
DP1-B1	F	5'-GGGATCCCCAGGAATTCCCCATATGAAGCCTCCTAAGGTAAAGAACGGT-3'
DP1-B2	F	5'-GGGATCCCCAGGAATTCCCCATATGCTCTATCGACGACAAAAGCCTCCACTGG-3'
DP1-B3	R	5'-GCTCGAGTCA GACATCCTCTATCCCCCTACCATGAGC-3'
DP1-B4	R	5'-GCTCGAGTCAGGGAACGTCTGGAAGGTAAAACTTG-3'
DP2	F	5'-GGGATCCCCAGGAATTCCCCATATGGAGCTTCCAAAGGAAATTGAGGAG-3'
	R	5'-GCTCGAGTCAGCGTTTGGAGAAGAAGTCGTCCAAGC-3'
ORF4	F	5'-GGGATCCATGGAGATTGTGTGGTGTGG-3'
	R	5'-GCTCGAGTCA GATTTCAAAACCC-3'
ORF5	F	5'-GGGATCCATGGTAAATACTGAGCTC-3'
	R	5'-GCTCGAGCTA ATCTTCGTTTTTTACATCCTCCAA-3'
Rad51 (*S. cerevisiae*)	F	5'-GCGGATCCATGGCTCAAGTTCAAGAACAA-3'
	R	5'-GCCTCGAGTACTCGTCTTCTTCTCTGG-3'

[a]F, forward primer; R, reverse primer.
[b]Restriction enzyme sites (*Bam*HI, *Xho*I and *Nco*I) are underlined.

MATERIALS AND METHODS

Yeast two-hybrid assay system

The two-hybrid system in the *Saccharomyces cerevisiae* EGY48 strain was used as described previously (16). The plasmids containing each gene in the operon were constructed in the pLexA and pB42 vectors (Clontech, Palo Alto, CA) by inserting the genes to fuse with the LexA DNA-binding domain and the GAL4 DNA activating domain, respectively.

Production and purification of the proteins

The gene for Orc1 was cloned into pFastBacHTb (Life Technologies Inc., Rockville, MD), which incorporated a 6×-Histidine tag at the N-terminus of the Orc1 protein. The designed *Bam*HI/*Xho*I sites were utilized to clone the gene for the Orc1 protein. All of the primers used to construct the vectors are shown in Table 1. All of the PCR amplifications described here were carried out from pPF1001, in which the entire operon is inserted into the pTV118N vector (Takara Shuzo, Kyoto, Japan) as described previously (10). The Orc1 protein was expressed in BTI-5B1-4 insect cells as described (17) except that the post-infection cultivation was performed at 18°C for 96 h. The genes for DP1, its deletion mutants, DP2, and DP1+DP2 were amplified by PCR using appropriate primers, respectively, and were inserted into the *Bam*HI/*Xho*I sites of the pGEX4T-2 vector plasmid to produce the target proteins as fusion with glutathione *S*-transferase (GST) (Pharmacia, Uppsala, Sweden). Cultures of *E.coli* JM109, carrying these plasmids, were grown at 37°C to an optical density at 600 nm of 0.5, and isopropyl-β-D-thiogalactoside (IPTG) was added to 1 mM to induce the expression of the target genes. The cell extracts were applied to a glutathione–Sepharose 4B column, and the GST was cleaved by the addition of thrombin protease (Pharmacia) to the fusion proteins bound to the column. The eluted proteins without GST were pure enough for use in biochemical assays. The gene for ORF4 was cloned into pMAL2c (New England Biolabs, Beverly, MA) and the protein fused with maltose binding protein (MBP) was produced in the same way as in the case of the GST-fusion proteins. The cell extract was applied to an amylose column (NEB). The fusion protein was eluted from the column with 10 mM maltose, and was digested with factor Xa protease (NEB). After heating the reaction mixture at 80°C for 20 min, followed by centrifugation, the supernatant was applied to a Sephacryl S-200 (Pharmacia) gel filtration column. The ORF4 protein was finally purified by HiTrapQ (Pharmacia) anion exchange column chromatography. The gene for RadB was cloned into pET21 (Novagen, Madison, WI) and the protein was purified as described previously (18), except the affi-gel heparin step was omitted. Purification of *P.furiosus* PolD (PolII) was described previously (10).

In vitro interaction

The cell lysate including the recombinant Orc1 protein was prepared as described above. The genes for ORF4 and RadB were cloned into the pTV118N vector, and the initiation codons of both genes were adjusted with the ATG sequence within the *Nco*I recognition site. *Escherichia coli* JM109 was then transformed with each plasmid. Production of the probe proteins was induced by the addition of IPTG to 1 mM, and the cells were incubated for a further 4 h. Insect cells or *E.coli* cells were harvested by centrifugation and were disrupted by sonication in buffer A [50 mM Tris–HCl, pH 8.0, 0.1 M NaCl, 2 mM 2-mercaptoethanol, and 10% (v/v) glycerol]. The soluble protein fraction was recovered by centrifugation for 20 min at 30 000 *g*, and 1 ml of the fraction from 15 ml of cell

culture was used for the pull-down assays. The GST fusion proteins (20 μg) were produced as described above and were immobilized to the glutathione–Sepharose 4B. After washing the glutathione–Sepharose resin with buffer A, the cell extracts, including each recombinant probe protein, were added and the mixture was incubated at 4°C overnight. The resins were washed again with TBST (0.1% Tween20 and 150 mM NaCl) eight times and the bound proteins were eluted with 60 μl of 10 mM glutathione in buffer A. The elution fractions were subjected to western blotting analysis.

Assay for DNA polymerization activities

The DNA polymerizing activities of DP1 mutants mixed with DP2 were measured by counting the incorporation of [methyl-^{3}H]TTP (Amersham) as described (19). The deletion mutants of DP1 were prepared as described above. The DP2 protein was prepared from a cell lysate of *E.coli* JM109 carrying pPFDP2, as described previously (10). The cells were harvested, washed with buffer A, and disrupted by sonication. The supernatant was incubated at 80°C for 30 min and the heat stable DP2 protein was recovered after the centrifugation. The supernatant was directly used for the DNA polymerization assay. To measure the relative activities, 0.15 μg of each DP1 mutant was used with the *E.coli* cell lysate including DP2.

Reticulocyte lysate expression and *in vitro* RadB binding assay with DP1 mutants

To produce the labeled DP1 deletion mutant proteins, RadA, RadB and *S.cerevisiae* Rad51 *in vitro*, their genes were cloned into pET21 using the *Nco*I–*Xho*I cloning sites shown in Table 1. These plasmids were purified using the Qiagen Midi-Prep system (Qiagen Inc., Chatsworth, CA), and then were transcribed and translated *in vitro* for 90 min at 30°C using the TNT T7 Coupled Reticulocyte Lysate system (Promega, Madison, WI) in the presence of L-[^{35}S]methionine (*in vitro* labeling grade, Amersham SJ1515) according to the manufacturer's instructions. The *radB* gene was cloned into pMAL2c in the same way as ORF4, as described above, and the RadB fusion with MBP was produced in *E.coli* JM109 cells. Fifty micrograms of MBP-RadB were immobilized to the amylose column, and 5–20 μl of *in vitro* translation products were used for the pull-down assay. The bound fractions were eluted with buffer A with 10 mM maltose, and the protein bands on the 12% SDS–PAGE were visualized by autoradiography.

Immunoprecipitation

Immunoprecipitations using the crude cell extract of *P.furiosus* were carried out as described (11). To immunoprecipitate the ^{35}S-labeled proteins with PolD (PolII), reticulocyte translations were performed as described above, and then aliquots (5–15 μl) were added to 100 μl of buffer A containing PolD (PolII) (0.3 μg). The amounts of the probe proteins were adjusted with reference to their translation levels. Anti-PolD (PolII) rabbit polyclonal anti-serum was added to each portion, and the reaction mixture was incubated with mixing for 1 h at 4°C. A 50 μl aliquot of 50% (v/v) protein A–Sepharose (Pharmacia) in buffer A was added, and the incubation was continued for an additional 1 h. The protein A–Sepharose beads were washed three times with 400 μl of PBS. After washing, 10 μl of loading buffer (5×) were added and the samples were boiled for 3 min. Twenty microliters of the supernatant were subjected to 12% SDS–PAGE. The proteins on the gel were visualized by autoradiography.

Measurement of primer extension ability

The primer extension ability was measured by following the previous study of *P.furiosus* PolD (PolII) (10). As the template–primer, poly(dA)$_{400}$·oligo(dT)$_{30}$ (Pharmacia) was used instead of the primer annealed single-stranded M13 DNA. The reaction mixture contained (in 25 μl) 20 mM Tris–HCl (pH 8.0), 1.5 mM $MgCl_2$, 2 mM each of dNTPs, and 0.1 mM ATP. The polymerase reactions were carried out at 70°C. Five microliters of the reaction mixture were removed at every 30 s after the initiation of the reaction and were suspended in 3 μl of stop solution (98% deionized formamide, 1 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue). Aliquots (2.5 μl) of each fraction were subjected to electrophoresis on 8% polyacrylamide gels containing 8 M urea.

RESULTS

Gene organization of the operon containing PolD genes in *P.furiosus*

Figure 1 shows the gene organization of the operon analyzed in this study. When we first reported this operon (10), we called the Rad51 homolog RadA, along with the other archaeal RecA/Rad51-like proteins found in *Methanococcus jannaschii*, *Haloferax volcanii* and *Sulfolobus solfataricus* (20). However, in subsequent reports of the two Rad51/Dmc1 homologs, they were called RadA and RadB (15,21). The sequence comparisons showed that our homolog is not RadA but RadB. Therefore, we started to call this protein RadB. In order to analyze the relationships and biochemical properties of the components in this operon, each gene was cloned, expressed, and purified independently, as described in the Materials and Methods. The results of the purification, as analyzed by SDS–PAGE, are shown in Figure 2. All of the proteins were produced in *E.coli* cells, except for the Orc1-like protein, which was produced using a baculovirus expression system. The recombinant Orc1-like protein was produced in a soluble form by an incubation of 5B1-4 cells at 18°C during the post-infection phase. Incubations at higher temperatures were less efficient in producing the protein in a soluble form.

DP1 and RadB interact in the two-hybrid system

To examine the interactions between the proteins in the operon, a two-hybrid analysis was performed (16,22). The genes for the tested proteins were fused to either the gene for the DNA-binding domain of the bacterial LexA protein or the yeast GAL4 transcriptional activator domain. After co-transformation of *S.cerevisiae* strain EGY48 carrying p8op-lacZ with each plasmid, we utilized a β-galactosidase assay to investigate the binding of the component proteins. The results of this experiment are shown in Figure 3 and are summarized in Table 2. Since we could not obtain the plasmids carrying the gene (*polC*) for DP2 due to the genomic instability, as seen with other DNA polymerases (23,24), the combinations with DP2 are not included. A distinct positive signal was obtained between DP1 and RadB in both combinations, with either protein was fused to LexA or Gal4. A positive signal was detected between DP1 fused to the LexA DNA-binding

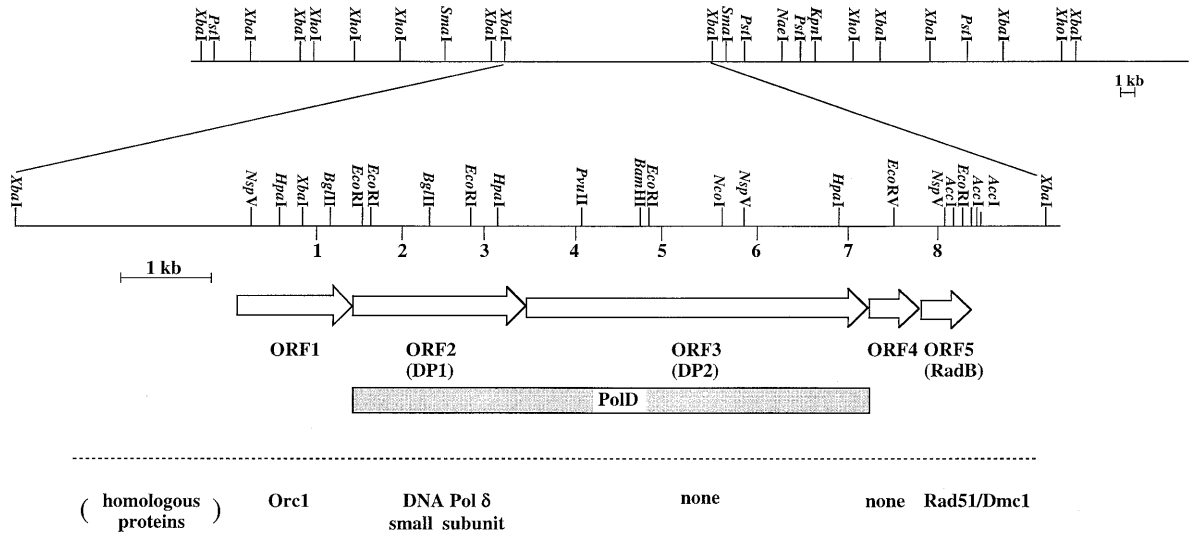

Figure 1. Gene organization and encoding proteins of the operon in *P.furiosus* analyzed in this study. The restriction map of the operon region is shown. Open arrows denote the ORFs. Eukaryotic proteins with similar sequences are indicated under each ORF.

Table 2. Analysis of *in vivo* protein–protein interactions

GAL4-activation domain fusion	LexA-binding domain fusion				
	ORF1 (Orc1)	ORF2 (DP1)	ORF4	ORF5 (RadB)	ctrl (vector)
ORF1 (Orc1)	ND	–	–	–	–
ORF2 (DP1)	+	+	–	+	–
ORF4	–	–	ND	–	–
ORF5 (RadB)	–	++	–	+	–
ctrl (vector)	–	–	–	–	–

Interactions of the two proteins in the yeast cells were quantified by assaying the β-galactosidase activities on the galactose-induced plates. ++ and + indicate that the colonies turned to blue within 12 and 24 h, respectively, after a starting incubation of 30°C, while – shows that the colonies remained white even after 24 h incubation. ND indicates that the combinations were not tested in this study.

domain and Orc1 fused to the Gal4 activation domain. However, no signal was detected from the combination of LexA-Orc1 and Gal4-DP1. Since the two-hybrid method sometimes detects indirect interactions (25), other biochemical methods are needed to confirm this specific physical interaction between the two proteins. The immunoprecipitation experiments showed that an anti-RadB antibody co-precipitated RadB and DP1 from the crude cell extract of *P.furiosus*. DP1 and Orc1 were never co-precipitated with either anti-DP1 or anti-Orc1 (Komori *et al.*, unpublished), thus supporting the specific interaction of DP1 and RadB.

In vitro interaction between DP1 and RadB

In order to test whether the interaction between DP1 and RadB is reproducible *in vitro*, a pull-down assay was performed using the recombinant proteins. DP1 fused to GST was produced in *E.coli* cells and was immobilized to glutathione–Sepharose 4B beads (Pharmacia). Cell extracts containing the other recombinant proteins, Orc1, ORF4 or RadB, were incubated with the immobilized proteins, and the unbound proteins were removed by washing. The proteins that bound specifically to the beads were detected by western blotting with each antibody

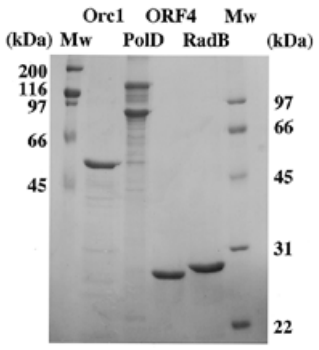

Figure 2. SDS–PAGE analysis of the recombinant proteins in the operon. The genes for each protein in the operon were cloned and expressed in *E.coli* or insect cells. The products, purified as described in the Materials and Methods, were subjected to SDS–PAGE and stained by Coomassie Brilliant Blue.

(Fig. 4). These experiments showed that RadB specifically interacts with DP1 *in vitro* (Fig. 4c, lane 3), in agreement with the results of the two-hybrid assay and the immunoprecipitation, as described above.

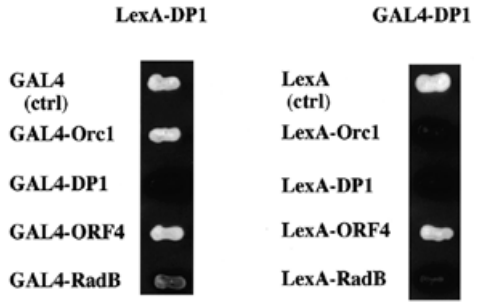

Figure 3. β-Galactosidase activity analysis in a two-hybrid assay. The interactions between DP1 and other proteins encoded in the operon were examined. The left slice shows the transformants using the plasmids producing DP1 fused with the LexA DNA-binding domain and the other proteins fused with the GAL4 DNA activating domain. The right slice shows the results from the opposite combinations. The results from the two-hybrid analysis performed in this study are summarized in Table 2.

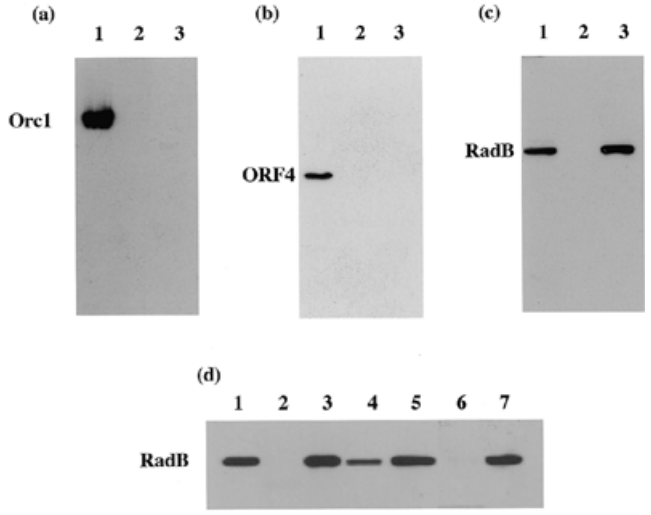

Figure 4. *In vitro* interactions between DP1 and other proteins encoded in the operon (**a**–**c**). In each panel, DP1 fused with GST was immobilized to glutathione–Sepharose beads, which were mixed with cell extracts containing each recombinant protein. The proteins bound to the column (lane 3) were eluted by glutathione and were analyzed by western blotting using anti-Orc1 (a), anti-ORF4 (b) and anti-RadB (c) serum. Purified recombinant proteins, Orc1, ORF4 and RadB, were loaded onto lane 1 of each panel. As a negative control, bound fractions with the immobilized GST alone were loaded onto lane 2. (**d**) *In vitro* interaction between RadB and PolD (PolII) subunits. DP1, DP2 and DP1+DP2 (their genes were cloned in tandem) fused with GST were immobilized to glutathione–Sepharose beads (lanes 3–5). *Escherichia coli* cell extracts containing RadB were mixed with the beads, which were then washed with TBST (0.15 M NaCl, 0.1% Tween20). The interacting proteins were eluted with 10 mM glutathione and were analyzed by SDS–PAGE followed by western blotting with anti-RadB serum. Lane 2 shows the fraction bound to the immobilized GST alone. Lane 6 shows the fraction bound to the immobilized PolBI (PolI). Purified RadB was loaded directly as positive marker (lanes 1 and 7).

To determine if DP2 makes a direct interaction with RadB, another *in vitro* experiment was carried out. The genes for DP2 and DP1+DP2 (their genes were cloned in tandem) were inserted into the pGEX vector and each GST-fusion protein, produced in *E.coli* cells, was immobilized onto the glutathione–

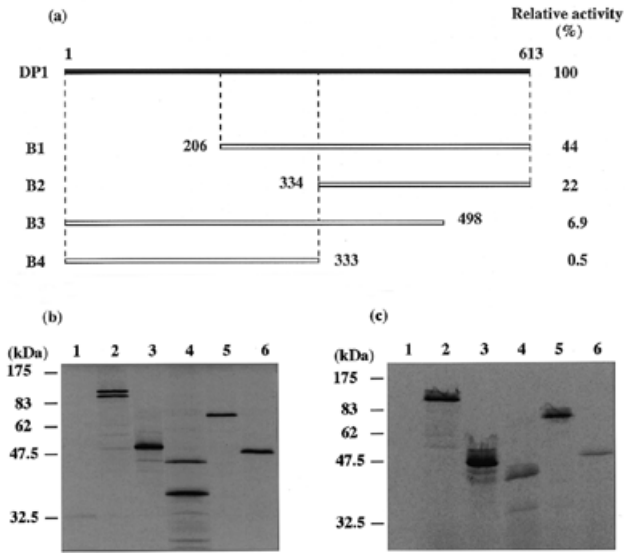

Figure 5. DP1 region analysis for binding with DP2 and RadB. (**a**) The diagrams show the deletion mutants of DP1, B1, B2, B3 and B4, with the numbers indicating the beginning and ending positions of the amino acids in DP1. The DNA polymerizing activities of the deletion mutants mixed with DP2 are indicated on the right relative to that of the intact DP1. (**b**) Expression of DP1 deletion mutant proteins *in vitro*. Rabbit reticulocyte lysates containing [^{35}S]methionine were programmed with either pET21a (vector control, lane 1), pETB.21 (wild type DP1, lane 2), or pETB1.21–pETB4.21 (B1–B4, lanes 3–6). The reaction products were then subjected to SDS–PAGE, and were autoradiographed. Equal amounts of probe proteins with the same radioactivities were loaded. Molecular weight standards are shown on the left. The 42 kDa band observed in lane 4 is derived from the background labeling of the rabbit reticulocyte lysate, because of the low level of translation of pETB2.21. (**c**) Pull-down assay of DP1 deletion mutants with RadB. RadB fused with maltose binding protein (MBP-RadB, ~15 μg) was immobilized on an amylose column. Equal amounts of each deletion mutant (5–15 μl) were added to the immobilized RadB, and bound proteins were eluted from the beads with 10 mM maltose. Aliquots were subjected to SDS–PAGE followed by autoradiography. The lanes correspond to those in (b).

Sepharose beads. The crude cell extract from *E.coli* producing RadB was loaded onto each immobilized column, and the bound fractions were subjected to the western blotting with the anti-RadB serum. This experiment showed that RadB interacts with both DP1 and DP2 (Fig. 4d, lanes 3 and 4). The signal intensity of the RadB band from the DP1-bound fraction (lane 3) was comparable to that from the (DP1+DP2)-bound fraction (lane 5), and both were stronger than that from the DP2-bound fraction (lane 4). This result suggests a preference to DP1 as a partner for the RadB protein. In contrast to the interaction with PolD (PolII), RadB did not bind with PolBI (PolI) under the same condition in this *in vitro* experiment (lane 6).

Domain analysis of DP1

In order to define the DP1 regions essential for the interactions with DP2 and RadB, we prepared four deletion mutants of DP1, as shown in Figure 5a. The region for the interaction with DP2 was examined by measuring the DNA polymerizing activity. All four deletion mutants of DP1 were produced as GST-fusion proteins in *E.coli*, according to the same procedure as that for the wild type DP1 protein. The PolII mutant proteins

were reconstituted *in vitro* by mixing the DP1 mutants with the *E.coli* cell extracts including the recombinant DP2, and their DNA polymerizing activities were measured by counting the incorporation of [^{3}H]dTTP into the activated calf thymus DNA. The assay was performed at a lower temperature (50°C), to accommodate the reduced thermostability of the DP1 mutants. A limited amount of DP2 was used with an excess of DP1 mutants. Significant DNA polymerizing activities were found only with the complexes bearing the C-terminal region of DP1 (Fig. 5a), consistent with the prediction from the sequence analysis of euryarchaeal DP1 (11).

To analyze the interaction between DP1 and RadB in more detail, the DP1 deletion mutant proteins were produced separately by an *in vitro* translation system, using rabbit reticulocyte lysates in the presence of ^{35}S-labeled methionine (Fig. 5b). Equal amounts of the labeled proteins were loaded onto an affinity column, on which the MBP-RadB fusion protein was immobilized to the amylose resin, to determine whether the DP1 mutants could be trapped on the column. The signal intensities of the mutant DP1 proteins shown in Figure 5c suggested that most of the B1 and B3 proteins were bound to the RadB-column; however, distinct amounts of B2 and B4 did not bind and were present in the column wash fractions. These results indicate that the DP1 region important for the interaction with DP2 and RadB lies at a different position, even though they may be overlapped. The C-terminal half (amino acids 334–613) is especially important for the PolD (PolII) complex (DP1+DP2) formation and the central part (amino acids 206–498) works mainly for the interaction with RadB.

The functional difference between the two RecA-like proteins, RadA and RadB

Pyrococcus furiosus has two Rad51/Dmc1-like proteins, RadA and RadB, as described above. We examined whether RadA also interacts with PolD (PolII) or if the interaction of PolD (PolII) with RadB is specific. Immunoprecipitation experiments were done using an *in vitro* translation mixture producing RadA, RadB, or *S.cerevisiae* Rad51 and an anti-PolD (PolII) antibody. Figure 6 shows that RadB, but neither RadA nor yeast Rad51, was precipitated with PolD (PolII), suggesting that RadB is a specific partner for PolD (PolII) among the Rad51/Dmc1-like proteins.

RadB does not affect the *in vitro* primer extension ability of PolD

To investigate the role of RadB in the complex with PolD (PolII), the DNA polymerizing activity of PolD (PolII) was measured in the presence or absence of RadB. Figure 7 shows the primer extension abilities of PolD (PolII) using poly(dA)$_{400}$·oligo(dT)$_{30}$. No significant difference in the PolD (PolII) activity was observed by the addition of RadB, as also found with the Orc1-like protein and ORF4. Since *P.furiosus* has a eukaryotic PCNA homolog (*Pfu*PCNA), which works as the elongation factor for PolD (PolII) (26), PCNA was added together with RadB in the reaction mixture. In this reaction with PolD (PolII) and *Pfu*PCNA, some inhibition of the elongation was observed in the presence of RadB. A preliminary immunoprecipitation experiment showed that RadB and *Pfu*PCNA do not have direct interaction with each other, and a gel-retardation assay showed that RadB has very strong binding affinity to DNA strands (data not shown). These results suggest that the binding of RadB to the template DNA may interfere the smooth sliding of *Pfu*PCNA on the DNA strands. Further detailed analyses are necessary to know the reason and meaning of the effect of RadB on the stimulation of elongation by *Pfu*PCNA.

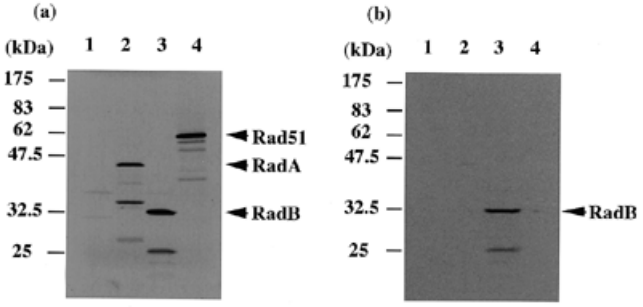

Figure 6. *In vitro* interaction of Rad51/Dmc1-like proteins with DP1. (**a**) Production of Rad51/Dmc1-like proteins *in vitro*. The genes for *P.furiosus* RadA, RadB and *S.cerevisiae* Rad51 were cloned into pET21a. The proteins produced *in vitro* by rabbit reticulocyte lysates containing [^{35}S]methionine were subjected to 15% SDS–PAGE and autoradiography (lanes 2, RadA; 3, RadB; 4, Rad51). The translation product from pET21a (no insert) is shown in lane 1 as a control. (**b**) Co-precipitation of Rad51/Dmc1-like proteins with recombinant PolD (PolII). Aliquots of lysates containing [^{35}S]methionine-labeled Rad51/Dmc1-like proteins (~15 µl) were mixed with 1 µg of PolD (PolII) and were co-precipitated with anti-PolD (PolII) serum. Immune complexes were then collected by protein A–Sepharose, washed extensively, and subjected to 15% SDS–PAGE. The protein bands were visualized by autoradiography. The lanes correspond to those in (a).

DISCUSSION

In this report, we have provided the evidence that DP1 and RadB specifically interact in *P.furiosus*. The DP1 protein is the second subunit of PolD (PolII), and is necessary for PolD (PolII) to exhibit extensive DNA synthesis activity by forming a complex with DP2 (10). Although the biochemical and sequence analyses of the DP2 protein showed that DP2 is the catalytic subunit of PolD (PolII), the actual function of DP1 is not known. DP1 and DP2 are conserved in the genomes of euryarchaeotes with known sequences (5–8), and the sequence comparison indicates that DP1 has significant similarity to the second subunit of eukaryotic Pol δ (11). It has been shown that the second subunit of Pol δ from mammalian cells is required for efficient stimulation of the polymerase processivity by PCNA (27,28). A recent report has concluded from the sequence similarity among the second subunit of Pol α, Pol δ and Pol ε, that they constitute a family of DNA polymerase-associated B subunits (29). Furthermore, in a significant contribution to cell-life, the second subunit of Pol α has been implicated in cell-cycle control (30,31), and the stability of the Pol ε complex, which is essential for chromosomal replication, depends on its second subunit (32).

RadB resembles proteins related to DNA recombination, in terms of its sequence similarity to the eukaryotic Rad51 and Dmc1 proteins. RadA proteins have been identified from other archaeal strains, and they have been shown to be functional and structural homologs of the RecA/Rad51 family (20,33,34). Moreover *Pk-REC*, a homolog of *P.furiosus* RadB from the

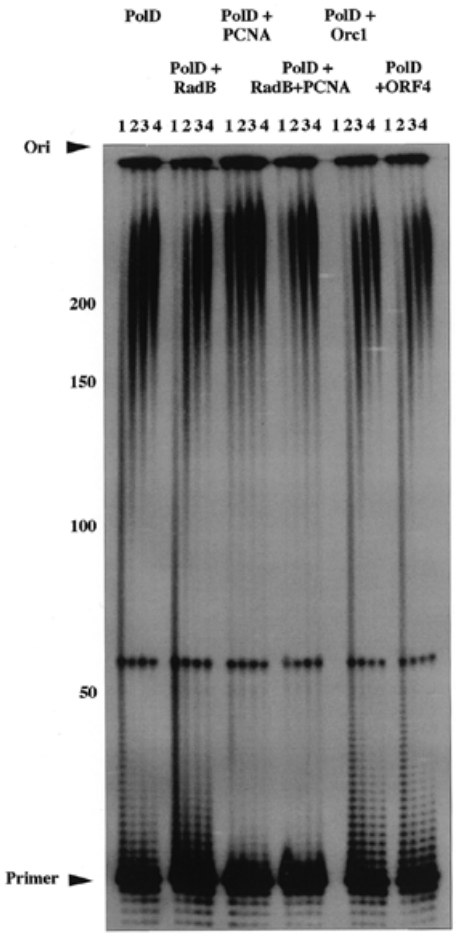

Figure 7. The chain elongation abilities of PolD (PolII) in the presence of other proteins encoded in the operon. A ^{32}P-labeled oligo(dT)$_{30}$ annealed to poly(dA)$_{400}$ was extended by PolD (PolII) alone or with the proteins indicated in the panel. Equal aliquots of the reaction mixture were removed at 30 (lane 1), 60 (lane 2), 90 (lane 3) and 120 s (lane 4) after the start of the reactions. The reaction products were subjected to a denaturing PAGE (8%) and were visualized by autoradiography. For each lane, 0.01 U of PolD (PolII) was used with either 0.3 µg of the other operon proteins or 1 µg *Pfu*PCNA.

Pyrococcus sp. KOD1 strain (63% identity to *Pfu* RadB) complements the UV sensitivity of an *E.coli recA* mutant (35). After the finding that the second RecA-like protein of *P.furiosus*, RadA, is more similar to the eukaryotic Rad51/Dmc1 and other archaeal RadA proteins, we confirmed that RadA, but not RadB, actually has DNA-dependent ATPase activity, D-loop formation activity, and strand exchange activity by itself (K.Komori, T.Miyata, I.Cann, I.Hayashi, J.DiRuggiero, K.Mayanagi, H.Shinagawa, K.Murikawa, F.Robb and Y. Ishino, unpublished). In our immunoprecipitation assay, RadA did not bind to PolD (PolII). An interesting question remains: what is the role of RadB?

The physical interaction demonstrated in this study (Figs 2–4) implied that RadB may be the possible third factor of PolD (PolII). Our trial, however, failed to show difference in the *in vitro* primer extension ability of PolD (PolII) in the presence or absence of RadB (Fig. 7). The amount of RadB in *P.furiosus* cells is predicted to be much less than that of the PolD (PolII) proteins, from the western blot analysis of the *P.furiosus* cell extracts (data not shown). There may be specific regulation of the *radB* gene expression or quick proteolysis of the produced RadB protein in *P.furiosus*. Although this stoichiometric difference indicates that the complex of PolD (PolII) and RadB does not always exist, once they are expressed under certain conditions, according to the operon order, they could interact and work together.

Is the interaction of DP1 and RadB commonly observed in Archaea? In the Pyrococcal DP1s, the important region for the binding with RadB is within the central part (amino acids 206–498), which comprises highly conserved (amino acids 334–498) and somewhat less-conserved regions (amino acids 206–334). On the other hand, the C-terminal region (amino acids 334–613) of the DP1, which is highly conserved among species, is essential for the interaction with DP2 (11,29). Moreover, the operon structure, including the genes for PolD (PolII) and RadB, is conserved amongst the Pyrococcals [*P.horikoshii* (8), *Pyrococcus abyssi*, personal communication from Dr Querellou, IFREMER, France) with known genomic sequences, while the other corresponding euryarchaeal genes are located separately. Further sequence observations and biochemical analyses will be necessary to determine whether the specific binding of the two proteins is observed in the archaeal cells. Pyrococcals are hyperthermophilic and grow optimally at 100°C, a condition requiring adaptations that affect all levels of the cellular machinery, including the enzymes that are involved in maintaining the integrity and stability of genomic DNA, and for this purpose, a highly efficient DNA repair system must be required (36–38). Our observation suggests that the Pyrococcals have a gene organization that forms a very effective replisome, in which RadB can work in replicational or recombinational repair with PolD (PolII).

It would be interesting to determine whether the interaction between a second subunit of DNA polymerase and a RecA/Rad51 family protein is common among organisms. Our two-hybrid analysis showed that *S.cerevisiae* Hys2, the second subunit of Pol δ, interacted with neither *S.cerevisiae* Dmc1 nor Rad51 (data not shown). Genetic studies, however, have provided some evidence for this: for over 20 years, it has been known that *recA polA* double mutants in *E.coli* are inviable (39,40). The amount of the proteins in the Rad52 recombinational repair pathway increases during the S phase in *S.cerevisiae* (41); POL3 (the catalytic subunit of *S.cerevisiae* Pol δ) plays an important role in the induced recombinational pathway (42,43); defects in Pol α and δ stimulate the level of the Holliday junction recombination intermediate in *S.cerevisiae* (44); and *S.cerevisiae* exhibits synthetic lethality when *Rad27*, which encodes the homolog of the 5′→3′ exonuclease function of *E.coli* PolI, is mutated together with genes related to homologous recombination including *Rad1*, *Rad50*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *MRE11*, *XRS2* and *RAD59* (45). In spite of these observations and our study, the question remains as to whether the replicational complex includes the factors involved in recombinational repair; does the RadB protein work as a recombinational protein in the replisome? Our present study suggests the possibility that DP1 could recruit the recombinational protein to the replicational machinery, or rather, DP1 could supply the 3′ ends of the DNA daughter strand to RadB. Indeed, in both in prokaryotes and eukaryotes,

3′ ends play a favored role in the initiation of recombination (46–49). Further study will be needed to answer these questions.

As the whole genome sequence data have suggested that the genome sizes of the thermophilic archaeal strains are smaller than those of other living eukaryotic organisms, some of the genes in eukaryotes do not exist in archaeal genomes, even though their mechanisms are similar to those of eukaryotes (5–8). The genomic size also affects the size of each gene or protein. From these observations, these archaeal organisms are predicted to have abbreviated molecular mechanisms for living phenomena in comparison with eukaryotes. Although the genetic strategy is limited thus far, the molecular biology of *P.furiosus* may lead to an understanding of the very complicated mechanisms of eukaryotic DNA replication and recombination.

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REFERENCES

1. Kornberg,A. and Baker,T.A. (1992) *DNA Replication.* 2nd Edn. W.H.Freeman and Co., New York, NY.
2. Brush,G.S. and Kelly,T.J. (1996) In DePamphilis,M.L. (ed.), *DNA Replication in Eukaryotic Cells.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1–43.
3. Waga,S. and Stillman,B. (1998) *Annu. Rev. Biochem.*, **67**, 721–751.
4. Woese,C.R. and Fox,G.E. (1977) *Proc. Natl Acad. Sci. USA*, **74**, 5088–5090.
5. Bult,C.J., White,O., Olsen,G.J., Zhou,L., Fleischmann,R.D., Sutton,G.G., Blake,J.A., FitzGerald,L.M., Clayton,R.A., Gocayne,J.D. *et al*. (1996) *Science*, **273**, 1058–1073.
6. Smith,D.R., Doucette-Stamm,L.A., Deloughery,C., Lee,H.-M., Dubois,J., Aldredge,T., Bashirzadeh,R., Blakely,D., Cook,R., Gilbert,K. *et al.* (1997) *J. Bacteriol.*, **179**, 7135–7155.
7. Klenk,H.P., Clayton,R.A., Tomb,J., White,O., Nelson,K.E., Ketchum,K.A., Dodson,R.J., Gwinn,M., Hickey,E.K., Peterson,E.J. *et al*. (1997) *Nature*, **390**, 364–370.
8. Kawarabayasi,Y., Sawada,M., Horikawa,H., Haikawa,Y., Hino,Y., Yamamoto,S., Sekine,M., Baba,S., Kosugi,H., Hosoyama,A. *et al*. (1998) *DNA Res.*, **5**, 55–76.
9. Uemori,T., Ishino,Y., Toh,H., Asada,K. and Kato,I. (1993) *Nucleic Acids Res.*, **21**, 259–265.
10. Uemori,T., Sato,Y., Kato,I., Doi,H. and Ishino,Y. (1997) *Genes Cells*, **2**, 499–512.
11. Cann,I.K.O., Komori,K., Toh,H., Kanai,S. and Ishino,Y. (1998) *Proc. Natl Acad. Sci. USA*, **95**, 14250–14255.
12. Cann,I.K.O. and Ishino,Y. (1999) *Genetics*, **152**, 1249–1267.
13. Ishino,Y. and Cann,I.K.O. (1998) *Genes Genet. Syst.*, **73**, 323–336.
14. Suckow,J.M., Amano,N., Ohfuku,Y., Kakinuma,J., Koike,H. and Suzuki,M. (1998) *FEBS Lett.*, **426**, 86–92.
15. DiRuggiero,J. and Robb,F.T. (1998) In Le,G.Y. and Halvorson,H. (eds), *New Developments in Marine Biotechnology*. Plenum Press, New York, NY, pp. 193–196.
16. Gyuris,J., Golemis,E., Chertkov,H. and Brent,R. (1993) *Cell*, **75**, 791–803.
17. Bell,S.P., Mitchell,J., Leber,J., Kobayashi,R. and Stillman,B. (1995) *Cell*, **83**, 563–568.
18. Benson,F.E., Stasiak,A. and West,S.C. (1994) *EMBO J.*, **13**, 5764–5771.
19. Ishino,Y., Iwasaki,H., Fukui,H., Mineno,J., Kato,I. and Shinagawa,H. (1992) *Biochimie*, **74**, 131–136.
20. Sandler,S.J., Satin,L.H., Samra,H.S. and Clark,A.J. (1996) *Nucleic Acids Res.*, **24**, 2125–2132.
21. Sandler,S.J., Hugenholtz,P., Schleper,C., Delong,E.F., Pace,N.R. and Clark,A.J. (1999) *J. Bacteriol.*, **181**, 907–915.
22. Fields,S. and Song,O. (1989) *Nature*, **340**, 245–246.
23. Boulet,A., Simon,M., Faye,G., Bauer,G.A. and Burgers,P.M.J. (1989) *EMBO J.*, **8**, 1849–1854.
24. Simon,M., Giot,L. and Faye,G. (1991) *EMBO J.*, **10**, 2165–2170.
25. Bartel,P., Chien,C.T., Sternglanz,R. and Fields,S. (1993) *Biotechniques*, **14**, 920–924.
26. Cann,I.K.O., Ishino,S., Hayashi,I., Komori,K., Toh,H., Morikawa,K. and Ishino,Y. (1999) *J. Bacteriol.*, **181**, 6591–6599.
27. Zhou,J.Q., He,H., Tan,C.K., Doweny,K.M. and So,A.G. (1997) *Nucleic Acids Res.*, **25**, 1094–1099.
28. Sun,Y., Jiang,Y., Zhang,P., Zhang,S.-J., Zhou,Y., Li,B.Q., Toomey,N.L. and Lee,M.Y.W.T. (1997) *J. Biol. Chem.*, **272**, 13013–13018.
29. Makiniemi,M., Pospiech,H., Kilpelainen,S., Jokela,M., Vihinen,M. and Syvaoja,J.E. (1999)*Trends Biochem. Sci.*, **24**, 14–16.
30. Araki,H., Hamatake,R.K., Johnston,L.H. and Sugino,A. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 4601–4605.
31. Nasheuer,H.-P., Moore,A., Wahl,A.F. and Wang,T.S. (1991) *J. Biol. Chem.*, **266**, 7893–7903.
32. Foiani,M., Marini,F., Gamba,D., Lucchini,G. and Plevani,P. (1994) *Mol. Cell. Biol.*, **14**, 923–933.
33. Woods,W.G. and Dyall-Smith,M.L. (1997) *Mol. Microbiol.*, **23**, 791–797.
34. Seitz,E.M., Brockman,J.P., Sandler,S.J., Clark,A.J. and Kowalczykowski,S.C. (1998) *Genes Dev.*, **12**, 1248–1253.
35. Rashid,N., Morikawa,M. and Imanaka,T. (1996) *Mol. Gen. Genet.*, **253**, 397–400.
36. DiRuggiero,J., Santangelo,N., Nackerdien,Z., Ravel,J. and Robb,F.T. (1997) *J. Bacteriol.*, **179**, 4643–4645.
37. Soares,D., Dahlke,I., Li,W.T., Sandman,K., Hethke,C., Thomm,M. and Reeve,J.N. (1998) *Extremophiles*, **2**, 75–81.
38. Grogan,D.W. (1998) *Mol. Microbiol.*, **28**, 1043–1049.
39. Gross,J.D., Grunstein,J. and Witkin,E.M. (1971) *J. Mol. Biol.*, **58**, 631–634.
40. Monk,M. and Kinross,J. (1972) *J. Bacteriol.*, **109**, 971–978.
41. Basile,G., Aker,M. and Mortimer,R.K. (1992) *Mol. Cell. Biol.*, **12**, 3235–3246.
42. Fabre,F., Boulet,A. and Faye,G. (1991) *Mol. Gen. Genet.*, **229**, 353–356.
43. Giot,L., Chanet,R., Simon,M., Facca,C. and Faye,G. (1997) *Genetics*, **146**, 1239–1251.
44. Zou,H. and Rothstein,R. (1997) *Cell*, **90**, 87–96.
45. Symington,L.S. (1998) *Nucleic Acids Res.*, **26**, 5589–5595.
46. Smith,G.R. (1987) *Annu. Rev. Genet.*, **21**, 179–201.
47. Sun,H., Treco,D. and Szostak,J.W. (1991) *Cell*, **64**, 1155–1161.
48. Silberstein,Z., Shalit,M. and Cohen,A. (1993) *Genetics*, **133**, 439–448.
49. Friedman-Ohana,R. and Cohen,A. (1998) *Proc. Natl Acad. Sci. USA*, **95**, 6909–6914.