DNA replication is a fundamental process that assures the maintenance of integrity of the genome. The various steps in DNA synthesis are basically similar among all organisms in eukaryota, bacteria, and archaea, although replication components differ. In eukaryotic cells, the final processing of Okazaki fragments on the lagging strand requires that the ribonucleotide portions as primers are completely removed by the concerted action of three nucleases, Fen-1, RNaseH, and Dna2. The resulting corresponding sections of the molecule are filled by DNA polymerases. The resulting nicks on the lagging strand are then sealed by DNA ligase (1–6). Dna2 from Saccharomyces cerevisiae encodes a 172-kDa protein that has single-stranded (ss) DNA-dependent ATPase, DNA helicase, and ssDNA-specific endonuclease activity (7, 8). The genetic and physical components differ. In eukaryotic cells, the final processing of Okazaki fragments on the lagging strand requires that the ribonucleotide portions as primers are completely removed by the concerted action of three nucleases, Fen-1, RNaseH, and Dna2. The resulting corresponding sections of the molecule are filled by DNA polymerases. The resulting nicks on the lagging strand are then sealed by DNA ligase (1–6). Dna2 from Saccharomyces cerevisiae encodes a 172-kDa protein that has single-stranded (ss) DNA-dependent ATPase, DNA helicase, and ssDNA-specific endonuclease activity (7, 8). The genetic and physical interactions of Dna2 with Rad27 (a yeast homologue of mammalian Fen-1) suggest that Dna2 plays a role in Okazaki fragment metabolism (9). The endonuclease activity associated with Dna2 preferentially cleaves ssDNA with free ends, and the helicase activity unwinds duplex DNA in the 5’ to 3’ direction. The cleavage reaction was stimulated by the presence of an RNA segment at the 5’-end of flap DNA. The 5’-end region of the Okazaki fragment is efficiently processed by Dna2 when it is displaced from the template by the DNA polymerase δ extending the upstream primer. These enzymatic properties of Dna2 provide a biochemical basis for a role in Okazaki fragment maturation (10, 11).

Archaea, the third domain of life, resemble bacteria in morphology and genomic organization (i.e. lack of a nucleus and a single circular genome). However, archaea and eukaryota likely have a common ancestor that is separated from bacteria (12, 13). Archaeal genome sequence analyses reveal that the cellular components for genetic processes such as DNA replication, transcription, and translation share many common features with eukaryota, whereas those for metabolic processes exhibit similarities to bacteria (14–18). Although the molecular mechanism of DNA replication in archaea seems to be a simplified version of the eukaryotic one, knowledge of archaeal DNA replication is still rudimentary (19–21).

The gene encoding the Dna2 homologue protein (PH0109) in Pyrococcus horikoshii (Dna2Pho) exists in an operon containing three other ORFs that are important in DNA replication. Although the gene encoding one ORF (PH0108) found upstream of the Dna2Pho gene is unknown, two other downstream ORFs (PH0112 and PH0113) are homologous to subunits of eukaryal replication factor C that load PCNA onto the template DNA. Moreover, the Dna2Pho gene is located only 9.5 kilobase pairs away from an operon adjacent to the replication origin, which consists of the genes encoding the Rad51 homologue (PH0119), small and large subunits of DNA polymerase D (PH0123 and PH0121), and origin recognition complex 1 (PH0124) (Fig. 1) (22–25). This clustering of genetically essential genes suggests that Dna2Pho possibly plays an important role in DNA replication similar to Dna2 in Eukaryota. In the present study, we report the first biochemical characterization of a Dna2 homologue from Archaea, the hyperthermophile P. horikoshii (Dna2Pho).

**EXPERIMENTAL PROCEDURES**

**Chemicals, Plasmids, Enzymes, and DNA Manipulation**—Ultracompetent Escherichia coli XL2-Blue MRF' cells and E. coli strain BL21-CodonPlus (DE3)-RIL competent cells were obtained from Stratagene (La Jolla, CA). The pET-21b vector was purchased from Novagen (Madison, WI). Vent DNA polymerase and KOD polymerase were purchased from New England Biolabs (Beverly, MA) and Toyobo (Osaka, Japan), respectively. Restriction enzymes were bought from Takara Shuzo (Kyoto, Japan) and Promega (Madison, WI) and used as recommended by the manufacturers. The ligation kit was purchased from Takara Shuzo and used according to the manufacturer’s directions. T4 polynucleotide kinase was obtained from Promega, while protease in-
Dna2 Homologue from Hyperthermophilic Archaeon P. horikoshii

| Table I  | Primers used in cloning and construction of the expression vectors |
|-----------|---------------------------------------------------------------|
| Primer    | Sequences and restriction sites in the primer               |
| P1        | 5'-TTTTAACCCTGAAACATATGCATATAGCTAGGCTACCTATAGGAAAGGATCCC-3' |
|           | NdeI                                                        |
| P2        | 5'-CTTACGAGGGCCGCAGAACGGTCCCGATCTCTCAGGCCCCACTGAC-3'        |
|           | NdeI                                                        |
| P3        | 5'-CTTCCGAGGTGGAATATATTTGGAGATTTACATTACCCAGG-3'            |
|           | NdeI                                                        |
| P4        | 5'-GCTTCTGTCGATCATATGAGCAGGAGGTG-3'                        |
|           | NheI                                                        |
| P5        | 5'-TTTTAGATCCCTCCTAGAATATTTGAG-3'                          |
|           | KpnI                                                        |
| P6        | 5'-AAAAAGATCCCCGCCATCTCTCCTCGAGCCCAC-3'                    |
|           | BamHI                                                      |
| P7        | 5'-TTTTGATCCCCCTCTAGAATATTTTGAG-3'                         |
|           | KpnI                                                        |

*The restriction sites are underlined.*

hibitor mixture tablets (EDTA-free) were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Chromosomal DNA of *P. horikoshii* OT3 was prepared from a sarcosyl lysate of the cells as described previously (26) with slight modification. The digestion of DNA with restriction enzymes and analysis of DNA fragments by agarose gel electrophoresis were performed under standard conditions (27). Transformation was carried out by the calcium chloride procedure (27). Mi-

teiscale preparation of *E. coli* plasmid DNA was performed by the alkaline lysis method (27) or with a QIAprep spin miniprep kit (Qiagen, Hilden, Germany). A QIAquick gel extraction kit (Qiagen) was used to recover DNA fragments from agarose gel.

**Cloning of the Genes and Construction of Expression Vectors** — The gene for Dna2Pho from *P. horikoshii* was amplified by polymerase chain reaction (PCR) with the primers P1 and P2 (Table I, Fig. 1). The amplified fragment was digested with *NdeI* and *SalI* and inserted into an expression vector pET-21b digested with *NdeI* and *BamHI*. The constructed plasmid was designated pET-Dna2PhoSs(21b). Since the Dna2 homologous Dna2Pab from *Pyrococcus abyssi* was longer by 122 amino acids at the NH₂-terminal than Dna2Pho, and another initiation codon TTG was found at the position of Dna2Pho corresponding to the initiation codon (GTC) of Dna2Pab, the ORF coding Dna2Pho was elongated by 122 amino acids. The elongated NH₂-terminal region was amplified by PCR with the primers PH0108/Dna2PhoL(21b). The sequences of the genes were verified using an ABI PRISM kit and model 310 capillary DNA sequencer (Applied Biosystems, Foster City, CA).

**Preparation of Radiolabeled Substrates for DNA Helicase and Nucleo-

tase Assay** — The oligomers used in this study (Table II) were labeled at the 5' end with T4 polynucleotide kinase and γ-[32P]ATP. The labeled oligonucleotide (5 pmol) was first heated with 2 μg of M13mp18 ssDNA or 98-mer oligonucleotide (5 pmol) in annealing buffer (20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and 50 mM NaCl) at 100 °C for 5 min, then kept at 67 °C for 1 h, and subsequently allowed to stand at 37 °C for 30 min (28). The annealing mixture was slowly cooled to room temperature. For the preparation of 3'-labeled substrate, the partial duplex substrate was prepared as described above and then labeled using the Klenow enzyme and α-[32P]dATP. 32P-Labeled oligonucleotide substrate was purified using a PCR purification kit (Qiagen). M13 DNA substrate was purified using a MicroSpin S-400 column (Amersham Biosciences) and PCR purification kit (Qiagen). To determine the direction of translocation, the 5'-labeled or 3'-labeled substrate was digested with *BamH I* at 30 °C for 6 h and purified using the PCR purification kit (Qiagen).

**ATPase Assay** — ATPase activity was measured in reaction mixtures (20 μl) containing 50 μM HEPES (pH 7.5), 1 μM dithiothreitol, 0.01% bovine serum albumin, 0.2 mM of [γ-32P]ATP (15 Ci/mmol), 1 mM MgCl₂, the indicated amount of polynucleotide, and the purified Dna2Pho helicase (30 ng). After incubation at 50 °C for 30 min, the reaction was terminated by adding 2 μl of 100 mM Na₂EDTA. An aliquot (2 μl) was spotted onto a polyethyleneimine-cellulose thin-layer plate. The reaction products (ATP, inorganic phosphate (Pₐ)) were separated by chromatography in a 1 m formic acid, 0.5 mM LiCl solution (29). The extent of ATP hydrolysis was quantified with the GS-525 molecular imager system (Bio-Rad).

**Helicase Assay** — The standard reaction mixture (20 μl) contained 50 mM HEPES (pH 7.5), 1 mM dithiothreitol, 0.01% bovine serum albumin, 2 μM ATP, 1 mM MgCl₂, and 0.1% poly(dA-dT)-labeled helicase substrate, and the purified Dna2Pho helicase. After incubation at 50 °C for 1 h, the reaction was terminated by adding 4 μl of a solution containing 50 mM Na₂EDTA, 0.5% SDS, 25% glycerol, and 0.025% bromphenol blue. The sample (10 μl) was loaded onto a 15% polyacrylamide gel in TBE buffer (89 mM Tris
Expression and Purification of Dna2Pho—According to the genomic sequence of *P. horikoshii*, the PH0109 gene encodes a protein of 1188 amino acids with a predicted molecular mass of 137 kDa. The PH0109 gene was first cloned into pET-21b to yield a construct encoding a fusion protein tagged at the COOH terminus with VEHHHHHHH (Fig. 1). The newly constructed plasmid was designated pET-Dna2PhoS(21b). Dna2Pho was expressed in the *E. coli* strain BL21-CodonPlus(DE3)-RIL. However, the majority of the protein was in insoluble fractions. The Dna2 homologue (Dna2Pab) from *P. abyssi* is longer by 124 amino acids at the NH₂-terminal region than Dna2Pho. Another initiation codon (TTG), corresponding to the initiation codon (GTG) of Dna2Pab, is located 366 nucleotides upstream from the initiation codon (GTG) of Dna2Pho. Therefore, the ORF coding Dna2Pho was elongated by 122 amino acids (Fig. 2). To obtain a soluble recombinant protein, the elongated Dna2Pho was inserted into pET-21b to yield a construct encoding a fusion protein tagged at the COOH terminus with VEHHHHHHH (predicted mass, 153 kDa, designated pET-Dna2PhoL(21b)) (Fig. 1). Although Dna2Pho protein was successfully expressed in the soluble fraction using pET-Dna2PhoL(21b), only a small amount was purified (data not shown). The gene encoding one ORF (PH0108) is found upstream of the Dna2Pho gene. Since the subunits of DNA polymerase (DP1Pho and DP2Pho) from *P. horikoshii* were successfully overexpressed in soluble fractions using a co-expression vector as previously described (22), to obtain large amounts of purified Dna2Pho, a co-expression vector designated pET-PH0108/Dna2Pho(21b) was constructed, although the function of PH0108 protein was unknown. In this vector, the PH0108 and Dna2Pho genes were connected in tandem into two ORFs. The two genes are transcribed under the control of a single transcription promoter and terminator (T7 promoter and T7 terminator). PH0108 and Dna2Pho are expressed as native and His-tagged forms of proteins, respectively (Fig. 1).

Both of them were successfully expressed in soluble fractions. Dna2Pho protein was purified in larger amounts using the co-expression system than with pET-Dna2PhoL(21b) as described under “Experimental Procedures.” Fig. 3 shows the SDS-PAGE of the purified sample. A major protein band of His-tagged Dna2Pho around 150 kDa and a minor protein band around 22 kDa were observed, whereas a band for PH0108 was not detected. The amino-terminal sequence of the 22 kDa protein was determined as MKVAKDLVVSL, which completely

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**RESULTS**

**Table II**

| Oligonucleotide | Sequence |
|-----------------|----------|
| 1               | 5’-GCA TGC CTA GAC GTC GAC TCT AGA GGA TCC CCC GGT ACC GAG CTC CAA TTA GTA ATG |
| 2               | 5’-GCT GCC GGA AAC CAG CCA AAG CGC CAT TCG-3' |
| 3               | 5’-GAA TAC AAG CTT GGG CTG CAG GTC GAT TCT AGA GGA TCC CCC GGC GAG CTC AAG TTA GTC GGA TTT |
| 4               | 5’-CTG GCT TAT CGA AAT TAA TAC GAC TCA CTA TAG GGA GAC CCC AAT TCG ACG TCG CCC GGC GAT |
| 5               | 5’-AGA GTC GAC CTA GCG CCA AGC CTA GTC TAT TC-3' |
| 6               | 5’-CTG GCT TAT CGA AAT TAA TAC GAC TCA CTA-3' |
| 7               | 5’-TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TAG AGT AGT CTA CCT GCA GCC CAA GCT |
| 8               | 5’-UUU UUU UUU UUU UUU UUU UUU UUU UUU UUU UUU UUU UUU UUU UUU UUU UUU UUU UUU UUU UUU UUU |

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**Experimental Procedures.**

Fig. 1. Schematic diagram showing the procedure for cloning and construction of the expression vectors for Dna2Pho from the genomic DNA of *P. horikoshii*. The primes are listed in Table I.

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agreed with the amino-terminal sequence of FKBP-type peptidyl-prolyl cis-trans-isomerase protein (FKBP-PPIase) (21 kDa) from E. coli (32), suggesting an intimate interaction between Dna2Pho and a chaperon-like protein, FKBP-PPIase. Since we could not separate Dna2Pho from FKBP-PPIase with any further purification steps, this protein sample was used for the experiments in this report. We also constructed a co-expression vector that produced PH0108 His-tagged at the COOH terminus and the native form of Dna2Pho. SDS-PAGE analysis showed that there was no band of Dna2Pho protein after nickel column chromatography (data not shown). These results may indicate that Dna2Pho does not interact with PH0108 or that FIG. 2. Conserved motifs among Dna2 homologues. The upper panel shows a schematic diagram of the Dna2 helicase from S. cerevisiae and P. horikoshii. The area boxed by diagonal striped squares shows the elongated amino-terminal region. The region boxed by black and vertical striped squares shows the RecB-like nuclease motif and helicase motifs, respectively. The number indicates the position of the amino acid from the amino terminus of the protein. The middle panel shows the nucleotide and the amino acid sequences of the elongated NH2-terminal region of Dna2 helicase from P. horikoshii. The amino acid sequence of Dna2 helicase in the data base of P. horikoshii starts from M. The lower panel shows the RecB-like nuclease motif and seven conserved helicase motifs (I, Ia, II, III, IV, V, and VI). The distances from the aligned regions to the protein termini and the distances between the conserved blocks, where more variable regions were omitted, are indicated. Identical amino acids are shown with a black background. Strictly conserved residues are boxed by black squares with asterisks. The abbreviations used are as follows: AddABsu, ATP-dependent nuclease subunit A from Bacillus subtilis (Swiss-Prot accession number P23478); Ex5BEco, exodeoxyribonuclease V β chain from E. coli (Swiss-Prot accession number P08394); Dna2Pho, Dna2 homologue from P. abyssi (Swiss-Prot accession number B75198); Dna2Pho, Dna2 homologue from P. horikoshii (C71231); Dna2Pho, Dna2 homologue from S. cerevisiae (Swiss-Prot accession number P38859); Dna2Spo, Dna2 homologue from Schizosaccharomyces pombe (Swiss-Prot accession number Q9URU2); Dna2Xla, Dna2 homologue from Xenopus laevis (Swiss-Prot accession number 2607285A).

Fig. 3. SDS-PAGE of the purified Dna2 homologue from P. horikoshii (Dna2Pho). M denotes molecular mass standards (150, 100, 75, 50, 35, and 25 kDa) purchased from Novegen. The numbers shown on the left of the figure indicate the sizes of the markers. The purified sample (0.5 μg of protein) was loaded onto the gel. The arrow indicates the band of FKBP-type peptidyl-prolyl cis-trans-isomerase co-purified with Dna2Pho.

Fig. 4. Dna2Pho has ATPase activity stimulated by single-stranded DNA. The upper panel shows DNA-stimulated ATPase activity. Dna2Pho (30 ng) was assayed for ATPase activity in the presence of closed circular single-stranded and double-stranded M13 DNA (5, 10, 20, 30, 40, and 50 fmol) at 50 °C for 30 min. The lower panel indicates ATPase activity plotted against the concentration of closed circular single-stranded M13 DNA (closed circle) or double-stranded M13 (open circle). The released Pi was quantified and calculated. The experiment was carried out three times, and the average of the three experiments is shown with error bars as indicated. The inset shows ATPase activity plotted against the addition of Dna2Pho into the reaction.
The amino acid residue corresponding to Pro504 of the Dna2Sce homologue gives a temperature-sensitive phenotype. Dna2Pho(20, 40, 60, 80, and 100 ng) was used in helicase assays. Oligonucleotide 1 (63-mers, Table II) labeled at the 5'- or 3'-end was annealed to M13 ssDNA and digested with Smal to produce linear substrates that were used to measure the polarity of displacement. The schematic structure of the substrate used is shown at the left of the figure. The asterisks indicate 32P-labeled ends.

The interaction between them is weak. Therefore, pET-PH0108/Dna2Pho(21b) was used to obtain a large amount of the purified Dna2Pho protein. It is not known what effect addition of the His tag may have had on the activities of Dna2Pho, although it is clear that the tagged protein has retained the DNA helicase and the nuclease activities.

**Primary Structure of Dna2Pho**—The alignment of sequences from Dna2Pho elongated by 122 amino acids, Dna2Pab, Dna2Sce, Dna2Spo, and Dna2Xla revealed many conserved residues over the entire length of the protein. Helicase and RecB-like nuclease motifs are found in Dna2 homologues as shown in Fig. 2 (33, 34, 35). The Dna2 P504S protein of *S. cerevisiae* gives a temperature-sensitive *in vivo* phenotype. This mutation affects ATPase, helicase, and nuclease activities. The amino acid residue corresponding to Pro504 of the Dna2Sce is not observed in Dna2Pho based on the alignment of amino acid sequences (data not shown), whereas Asp142, Glu155, and Tyr173 are found in the RecB-like motif of Dna2Pho equivalent to the catalytic residues of Dna2, Asp657, Glu675, and Tyr693, respectively (36, 37).

The cysteine cluster conserved among eukaryotic Dna2 proteins is also observed in archaeal Dna2 homologues. However, the function of this cluster is still unknown (35). Dna2Pho possesses seven typical helicase motifs (I, Ia, II, III, IV, V, and VI) (33, 34). The sequence (GTGKT) in the walker A box, the NTP binding motif within motif I as shown in Fig. 2, is completely conserved. Although many residues are conserved within RecB-like nuclease and helicase motifs, no conserved residues were identified in other regions. Dna2Pho possesses a RecB-like nuclease motif and helicase motifs. However, the location of these motifs in Dna2Pho is different from that in Dna2Sce (Fig. 2, upper panel).

**ATPase Activity Stimulated by a Single-stranded DNA**—Since the inset in Fig. 4 shows that Dna2Pho hydrolyzed ATP in a dose-dependent manner, Dna2Pho (30 ng) was added to the reaction mixture for the ATPase assay. Purified Dna2Pho (30 ng) was assayed for ATPase activity in the presence of increasing concentrations of closed circular single- and double-stranded (ds) M13 DNA. Although the optimal growth temperature of *P. horikoshii* is 95 °C, ATPase assays were performed at 50 °C because portions of double-stranded DNA are likely to denature at high temperature. The hydrolysis of ATP by the Dna2 from *S. cerevisiae* is markedly stimulated by ssDNA (8). ATPase activity of Dna2Pho was also stimulated as the amount of ssDNA increased. By the addition of 40 fmol of M13 ssDNA, the ATPase activity was stimulated ~10-fold; however, ATPase activity was not stimulated by dsDNA. These results indicate that Dna2Pho has ATPase activity stimulated by single-stranded DNA (Fig. 4).

**Nuclease and DNA Helicase Activities of Dna2Pho Need Both ATP and MgCl2**—To confirm the existence of helicase and nuclease activities, an M13 single-stranded DNA circle hybridized to a 30-nt oligonucleotide was used as a substrate. As shown in Fig. 5, no bands due to the displacement or degradation of substrate were observed on varying the MgCl2 concentrations (5 and 10 mM) than ATP (2 mM). Neither helicase nor nuclease activity was detected when EDTA was added to the reaction mixture. These activities were not detected when the reactions were performed with the protein sample purified with the cell extracts of transformants harboring pET-21b vector alone by the same purification procedure. The results indicate that Dna2Pho possesses nuclease and ATP-dependent DNA helicase activities and Mg2+ ion is required for both.

An ATP-dependent DNA Helicase That Unwinds DNA in the 5’ to 3’ Direction—Two classes of DNA helicase have been reported. DnaB translocates in the 5’ to 3’ direction (32); however, minichromosome maintenance protein translocates in the opposite direction (from 3’ to 5’) (29, 38–40). In this experiment, the DNA unwinding polarity of Dna2Pho was determined by blocking the nuclease activity using high ratios of ATP (2 mM) to Mg2+ (1 mM). The partial duplex DNA sub-
would displace the 5'-labeled fragment from the substrate. In procedures, "top substrates used are shown at the top of the figures. The asterisks indicate 32P-labeled ends. The oligonucleotides used to construct each substrate are indicated as circled numbers that are listed in Table II. M denotes molecular size markers prepared by labeling a synthetic mixture (2, 3, 4, 6, 40, 50, and 63-mers) and commercial size markers (dgATC)\_n, where n denotes oligonucleotides 8-32-mers in length, Amersham Biosciences) at their 5'-ends with T4 polynucleotide kinase. The numbers shown on the left of the figures indicate the size of the markers. The reaction mixtures contained 50 mM HEPES (pH 7.5), 1 mM dithiothreitol, 0.01% bovine serum albumin, 2 mM ATP, 10 mM MgCl\_2, the 32P-labeled substrate as described under "Experimental Procedures," and Dna2Pho (130 ng in a 20 μl-reaction mixture), at 50 °C. Samples were removed (0, 5, 10, 20, 40, 60, and 120 min), quenched, and loaded on a 15% denaturing polyacrylamide gel (7 M urea). The gel was dried, and the radioactivity was visualized using the GS-525 molecular imager system. A, the nuclease activity of Dna2Pho using single-stranded DNA as substrate. B, the nuclease activity of Dna2Pho using two partial duplex DNA (5'-overhang and 3'-overhang) as substrate.

strates, consisting of linear M13 ssDNA containing either 3'-labeled or 5'-labeled 32-nucleotide (nt) fragments at both ends, were prepared by digesting with SmaI as shown in Fig. 6 and under "Experimental Procedures." Since the substrates comprise duplex regions at both ends of a long linear molecule, Dna2Pho must first bind to the internal single-stranded regions of these substrates. If the enzyme subsequently moves from the 3' to 5' along the single-stranded DNA segment, it would displace the 5'-labeled fragment from the substrate. In contrast, the 3'-labeled fragment would be displaced, if the enzyme migrates in a 5' to 3' direction.

As shown in Fig. 6, the 3'-labeled fragment was displaced gradually from the substrate as the addition of Dna2Pho to the reaction mixture increased, whereas little displaced 5'-labeled fragment was observed. The displaced fragments were quantified and the values were normalized as described under "Experimental Procedures." The value of DNA helicase activity was more than 70% (closed circle) using a 3'-labeled substrate, while less than 12% (open circle) using a 5'-labeled substrate when Dna2Pho (100 ng) was added to the reaction mixture. When a 30-mer duplex fragment, which was blunt-ended at both ends, was assayed, the value of DNA helicase activity increased gradually as the amount of Dna2Pho in the reaction increased. The helicase activity was −18% when Dna2Pho (120 ng) was added to the reaction mixture (data not shown). These results suggest that the DNA helicase activity using a 5'-labeled substrate was due to movement of the enzyme in the 5' to 3' direction from the blunt end; hence, Dna2Pho translocates in the 5' to 3' direction, similarly to the yeast Dna2 (10).

Dna2Pho Has Nuclease Activity That Prefers the Free 5'-End to 3'-End—To identify the nuclease activity of Dna2Pho, single-stranded, double-stranded, 3'-overhang, and 5'-overhang DNA were used as substrates at high ratios of Mg\(^{2+}\) (10 mM) to ATP (2 mM). As shown in Fig. 7A, the single-stranded DNA substrate was rapidly degraded to oligonucleotides varying in size with the time course of reaction, whereas the double-stranded DNA (dsDNA) substrate was degraded slowly. The trinucleotide observed using dsDNA substrate might be due to unwinding from the 5'-overhang end and subsequently cleavage of the translocating side of dsDNA. These results demonstrate that the nuclease activity of Dna2Pho prefers ssDNA. When the 3'-overhang substrate was used for nuclease assay, the nuclease activity was decreased, compared with the activity against the single-stranded DNA (Fig. 7, A and B). On the other hand, the 5'-overhang DNA substrate was more efficiently degraded than the 3'-overhang DNA substrate, indicating that for nuclease activity, Dna2Pho is likely to prefer ssDNA with free 5'-end as substrate (Fig. 7, A and B).
Both Nuclease and Helicase Activities Were Inhibited by RNA/DNA Chimeric Substrate—The endonuclease activity of Dna2 from *S. cerevisiae* is stimulated by substrate with an RNA segment at the 5′-end, and the helicase activity enhances the endonuclease activity. Therefore it is considered that Dna2 removes RNA-DNA primers of Okazaki fragments with both unwinding and cleavage activity coupled to each other during Okazaki fragment processing (10, 11, 41).

To investigate the effect of RNA/DNA chimeric substrate on the activities, a Y-structured substrate containing a 12-nt oligonucleotide (U) segment at its 5′-end was prepared as shown in Fig. 8. In the DNA helicase assay, displaced fragments were observed as the Dna2Pho concentration increased. However, no displaced fragments were produced from the substrate with an RNA segment at the 5′-end, indicating that Dna2Pho could not unwind the substrate (Fig. 8A).

The nuclelease activity was assayed on single-stranded DNA or single-stranded RNA/DNA chimera, respectively. As shown in Fig. 8B, the single-stranded DNA was degraded into pieces varying from 50-mer to the size of a monomer, whereas RNA-DNA oligonucleotide was not digested by the nuclelease activity. The Y-structured DNA substrate was also degraded by Dna2Pho just like single-stranded DNA. However, the Y-structured RNA-DNA chimeric substrate was not degraded by the nuclelease activity. These results demonstrate that Dna2Pho can not displace and cleave substrates with an RNA segment at the 5′-end and that the 5′-end moity of ssDNA is important for the recognition or binding of Dna2Pho to the substrate.

**DISCUSSION**

In this report, we have identified a number of biochemical properties of the Dna2Pho from *P. horikoshii*. The gene for Dna2Pho is encoded in an operon, in which two subunits of replication factor C are also present. Moreover, the Dna2Pho gene is located only 9.5 kilobase pairs away from an operon adjacent to the replication origin, which consists of genes encoding a Rad51 homologue, small and large subunits of DNA polymerase D, and origin recognition complex 1 (14, 15, 23, 24). This clustering of genetically essential genes indicates that the gene is located only 9.5 kilobase pairs away from an operon adjacent to the replication origin, which consists of genes encoding the short RNA segment, typically 8–12 nucleotides in length (43). The primase complex of the hyperthermophilic archaeon, *Pyrococcus furiosus*, has been characterized by Liu et al. (44). The amino acid sequences of two subunits, Pfp41 and Pfp46, have similarity to p48 and p58 of the eukaryotic primase complex, respectively. The p41-p46 complex can synthesize both DNA (~700 bases in length) and RNA primers (12–40 nucleotides in length), whereas the DNA primase from yeast cannot synthesize DNA primers. We also characterized the DNA primase from *P. horikoshii* (45). Surprisingly, the complex could synthesize long DNA primers 10 times more effectively than RNA primers and also could synthesize DNA/RNA hybrids. Liu et al. (44) speculated that the primer synthesis was started by RNA using ATP in *P. furiosus*, because the p41-p46 complex can discriminate ATP from other NTPs. We used a substrate with a pol(yU)_{12} segment at its 5′-end in this report; therefore, further experiments to elucidate specificity against a substrate with a pol(A) segment at the 5′-end might be necessary. Furthermore, the isolation and characterization of Okazaki fragments from *Pyrococcus* cells are very interesting, since it is still unclear whether DNA primers are synthesized in vivo or not. If the DNA primase of *Pyrococcus* species could synthesize a DNA primer in vitro, the properties of Dna2Pho might be suitable for Okazaki fragment processing in *Pyrococcus* cells.

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