Identification of a Novel Helicase Activity Unwinding Branched DNAs from the Hyperthermophilic Archaeon, *Pyrococcus furiosus*

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To identify the branch migration activity in archaea, we fractionated *Pyrococcus furiosus* cell extracts by several chromatography and assayed for ATP-dependent resolution of synthetic Holliday junctions. The target activity was identified in the column fractions, and the optimal reaction conditions for the branch migration activity were determined using the partially purified fraction. We successfully cloned the corresponding gene by screening a heat-stable protein library made by *P. furiosus* genomic DNA. The gene, *hjm* (Holliday junction migration), encodes a protein composed of 720 amino acids. The Hjm protein is conserved in *Archaeabeta* and belongs to the helicase superfamily 2. A homology search revealed that Hjm shares sequence similarity with the human Polθ, HEL308, and *Drosophila* Mus308 proteins, which are involved in a DNA repair, whereas no similar sequences were found in bacteria and yeast. The Hjm helicase may play a central role in the repair systems of organisms living in extreme environments.

Archaea, the third domain of life (11), is distinct from both Bacteria and Eukarya. Archaea share many similarities with Eukarya in their genetic information processing pathways, including DNA replication, transcription, and translation (12), although cellular structure is prokaryotic. Therefore, the archaean processes provide a useful model systems to understand the much more complex mechanisms of their eukaryotic equivalents. Proteins involved in homologous recombination are also conserved between Archaea and Eukarya. We characterized the *Pyrococcus furiosus* RadA and RadB proteins (13–15), which play a central role in the initiation of homologous recombination. These archaean recombinases have sequences more similar to that of eukaryotic Rad51 than bacterial RecA (16, 17). In addition, we showed that the *P. furiosus* RPA, which is composed of three subunits, RPA41, RPA14, and RPA32, like the eukaryotic RPA (p70-p14-p32), but different from bacterial SSB (homotetramer), clearly stimulated a RadA-mediated strand exchange reaction (18). The homologs of the eukaryotic Rad50 and Mre11 proteins, which may work in the very early steps of homologous recombination, after double-stranded break repair, are also conserved in Archaea (19, 20). Regarding the late stage, in which the HJ intermediates are processed, we identified an archaeal HJ resolvasse and named it Hjc (21). Contrary to our expectations, Hjc is an archaeae-specific protein, and neither its sequence nor its three-dimensional structure is similar to other known HJ resolvases (22, 23). Therefore, the HJ resolvasse is quite interesting, from an evolutionary point of view (24).

In our series of experiments, we fractionated *P. furiosus* cell extracts by several chromatography steps and assayed for the ATP-dependent resolution of synthetic Holliday junctions to identify the activity for HJ branch migration in archaea. The target activity was identified in the column fractions, and the optimal reaction conditions for the activity were determined, using the partially purified fraction. Then we succeeded in cloning the corresponding gene by screening a heat-stable protein library of *P. furiosus*. The gene, *hjm* (Holliday junction migration), encodes Hjm, which is composed of 720 amino acids. The Hjm protein is conserved in Archaea, and there are some eukaryotic proteins sharing sequence similarity to Hjm. The physiological functions of Hjm in archaeal cells and the evolution of the Hjm protein are very interesting themes to consider.

**EXPERIMENTAL PROCEDURES**

**Synthetic DNA Substrates—Oligonucleotides with the following sequences were synthesized: sequence 1, 5'-GTGACCGTCTCCGGGACCG-TGCATGTGCTCAGAGGTTTTTACCCGTCACCAAGGAAACCGCCGGAG-ACGAAAAGG-3'; sequence 2, 5'-CCCTTTGTCCTGCCGGGCTTGTCCGGA-TGACGTTGAACACCTTCTGACACATGGCCAGCCCCTGACACCCCGCC-C-3'; sequence 3, 5'-TGGCCGGTGTCCGGGCTCAGCATGGTGTCAG-3'.**

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buffer A containing 1M ammonium sulfate, and the proteins were precipitated with ammonium sulfate (80% saturation). The precipitated proteins were resuspended in 40 mM Tris-acetate, pH 7.8, and 0.5 mM magnesium acetate. Four-way junctions with specific structures were prepared by annealing the appropriate oligonucleotides in TAM buffer, containing 40 mM Tris-acetate, pH 7.8, and 0.5 mM magnesium acetate. After centrifugation, the precipitate was resuspended in 300 mM potassium phosphate buffer containing 0.15% ammonium sulfate, and centrifuged at 30,000 × g for 60 min at 4 °C. The cell-free extract was treated with polyethyleneimine (final concentration, 0.15%) and centrifuged at 30,000 × g for 15 min at 4 °C. After dialysis against buffer A, the precipitated proteins were precipitated with ammonium sulfate (80% saturation). The precipitated proteins were resuspended in 60 mM of buffer A containing 2 mM DTT, and the eluted proteins were dialyzed against buffer A containing 0.5 mM NaCl. After dialysis, the supernatant was diluted with 2.5 volumes of 0.32–0.37 M NaCl. The eluted protein was pooled and stored at 4 °C.

Partial Purification of the Branch Migration Activity from P. furiosus Cells—P. furiosus cells (DSM 3838 strain) were activated as described earlier (27), and 52 g of cells were obtained. The cells were disrupted by sonication in 500 mM buffer A (50 mM Tris-acetate, pH 8.0, 0.5 mM diithiothreitol, 0.1 mM EDTA, and 10% glycerol) containing 1 mM phenylmethylsulfonyl fluoride. After centrifugation at 100,000 × g for 60 min at 4 °C, the cell-free extract was treated with polyethyleneimine (final concentration, 0.15%) and centrifuged at 30,000 × g for 15 min at 4 °C. After dialysis against buffer A, the precipitated proteins were precipitated with ammonium sulfate (80% saturation). The precipitated proteins were resuspended in 60 mM of buffer A containing 2 mM DTT, and the eluted proteins were dialyzed against buffer A containing 0.5 mM NaCl. After dialysis, the supernatant was diluted with 2.5 volumes of 0.32–0.37 M NaCl. The eluted protein was pooled and stored at 4 °C.

Western Blot Analysis—P. furiosus cells (1 g) were disrupted by sonication in 15 mL of buffer B containing proteinase inhibitor (Complete Mini) and 1 mM phenylmethylsulfonyl fluoride. After centrifugation at 100,000 × g for 60 min at 4 °C, the cell-free extract was treated with polyethyleneimine (final concentration, 0.15%) and centrifuged at 30,000 × g for 15 min at 4 °C. After dialysis against buffer A, the precipitated proteins were precipitated with ammonium sulfate (80% saturation). The precipitated proteins were resuspended in 60 mL of buffer A containing 2 mM DTT, and the eluted proteins were dialyzed against buffer A containing 0.5 mM NaCl. After dialysis, the supernatant was diluted with 2.5 volumes of 0.32–0.37 M NaCl. The eluted protein was pooled and stored at 4 °C.

Gel Filtration and Glyceraldehydr Gradient Centrifugation—Gel filtration chromatography was performed with the SMART system (Amersham Biosciences). The MonoS fraction (fraction number 16) or purified recombinant Hjm protein was applied to a Superdex 200 3.2/30 column (Amersham Biosciences), pre-equilibrated with buffer E (50 mM Tris-acetate, pH 8.0, 0.5 mM EDTA, 1 mM DTT, and 0.3 mM NaCl). The eluted fractions were subjected to a branch migration assay, as described previously (10). The branch migration activity was estimated from the elution profiles of standard marker proteins, including thyroglobulin (670,000), γ-globulin (158,000), ovalbumin (44,000), and myoglobin (17,000). Glyceraldehyde gradient centrifugation was performed as described earlier (28). The purified Hjm protein (100 μg) was sedimented through a 10-mL continuous 10–35% (v/v) glycerol gradient by 40,000 rpm at 4 °C for 24 h in a Beckman SW41 rotor. The standard marker proteins as described above were sedimented under identical conditions.

Branch Migration of Recombination Intermediates—The recombination intermediates were produced by in vitro strand exchange reaction using plasmid DNAs, as described previously (21, 29). The single-stranded circular pUC118 DNA and the Kas-PstI-digested double-stranded pUC118 were mixed to produce a gapped DNA, and the gapped DNA (6 μM as nucleotide concentration) was preincubated with the pE. coli RecA protein (2.7 μM) in a buffer containing 20 mM Tris-acetate, pH 7.5, 15 mM MgCl2, 2 mM DTT, 2 mM ATP, and 100 μM bovine serum albumin at 37 °C for 5 min for the strand exchange reaction. To produce the a-stranded DNA, recombination intermediates, 3′-2′P-labeled linear DNA (PstI-digested) was added to 3 μM. After 15 min of reaction, the reaction mixture was deproteinized by phenol extraction. Purified Hjm protein was added to 100 nM to the solution containing a-stranded DNA in 10 mM Tris-acetate, pH 8.0, 2 mM ATP, 5 mM MgCl2, and 1 mM DTT and was incubated at 55 °C for 0–60 min. The reaction products were deproteinized by incubation with protease K followed by phenol extraction and were separated by 12% agarose gel electrophoresis in TAE buffer. Detection of the products was performed by autoradiography.
Computer Analysis of the Amino Acid Sequences—Search for the homologous sequences in the databases with BLAST was carried out at a website (www.ncbi.nlm.nih.gov/cgi-bin/BLAST/). The gene corresponding to hjm has been registered as the gene number of PF0677 in the P. furiosus genome data base. The hjm homologs in the other archaeal genomes correspond to the gene numbers, PAB0592 (Pyrococcus abyssi), AF22454 (Archaeoglobus fulgidus), Halorad24b (Halobacterium salinarum), MTH810 (Methanothermobacter thermoautotrophicus), PAAE0594 (Pyrolobus aerophilus), SSO02462 (Sulfobolus solfataricus), STO 0590 (Sulfobolus tokodaii), and APE0191 (Aquopyrum pernix) in each genome data base. The sequences of human DNA polymerase 6, Hel308, and Drosophila melanogaster Mus308 were derived from the genes with accession numbers AAK39635, NP_598375, and AAB67306, respectively.

RESULTS

Identification and Partial Purification of the Branch Migration Activity—We tried to identify the branch migration activity corresponding to that from the bacterial RuvB protein in P. furiosus cells. The total cell extracts of P. furiosus were fractionated by anion exchange (HiTrap Q) chromatography, and the target activity was assayed using a 32P-labeled synthetic HJ. However, the branch migration activity to produce splayed arm DNAs was not detected in any fraction. Then polyethyleneimine precipitation was done to concentrate the nucleic acid-binding proteins before the ammonium sulfate precipitation and the column chromatography as described under “Experimental Procedures.” These procedures were effective, and the branch migration activity eluted during the elution of the adex resin, because chromatographic profile showed that the elution peak of Hjm was not symmetric but had some tailing.

Biochemical Characterization of Hjm—Using purified Hjm protein, its HJ unwinding activity was characterized in more detail. Hjm dissociated the synthetic HJ, HSL DNA, to two splayed arms by a branch migration-dependent manner in vitro as shown in Fig. 3A. Therms thermophilus RuvB and RuvA-RuvB complex were used for the same assay to compare the efficiencies of the unwinding reaction. The Hjm dissociated the synthetic HJ, HSL DNA, to two splayed arms at lower concentrations (3–10 mM). In the higher concentrations (>10 mM), Hjm dissociated HSL DNA very efficiently, and in these cases, approximately half of the splayed arms DNA was dissociated to single-stranded DNAs (Fig. 3). In the same unwinding assay, T. thermophilus RuvAB, with 200 mM as RuvB monomer concentration, dissociated 34% of HSLS to two splayed arms in 30 min. With the same concentration of the proteins, Hjm showed more efficient dissociation of HSL DNA (Fig. 3B). This dissociating activity was ATP-dependent (Fig. 4A). However, some reaction products were observed in the reactions with ADP and ATP.*S. Further investigations should be done to confirm that these nucleotides actually work for the reaction. The reaction was most efficient with an ATP concentration of at ~5–10 mM (Fig. 4A). Interestingly, the HJ were dissociated to single-stranded DNA with increasing concentrations of ATP. A divalent cation was also essential for the reaction. The optimal temperature of the dissociation reaction, and Mg2+ worked most efficiently among five kinds of metal cations (Fig. 4B). In the case of MgCl2, the reaction progressed well with less than 10 mM MgCl2 (Fig. 4C). This reaction dissolving activity was quite sensitive to the salt concentration, because the reaction was inhibited with increasing concentrations of NaCl (Fig. 4D). In addition to these properties, Hjm is very heat-stable. No activity was lost even after an incubation of the protein at 98 °C for 90 min (data not shown). The optimal temperature of the reaction was observed at least at 80 °C (data not shown).

Hjm-induced Branch Migration of RecA-mediated Recombination Intermediates—To investigate whether the Hjm protein has branch migration activity with a natural recombinational intermediate, we prepared the Holliday junction by using a RecA-mediated strand exchange reaction between gapped circular pUC18 and homologous linear duplex DNA labeled at its 3'-termini with 32P, as shown in Fig. 5A. The recombination intermediate, called an α-structured DNA, can be dissolved to nicked circular and linear duplex DNA by a branch migration junction migration). Using the highly purified Hjm protein, a polyclonal antibody was prepared. Western blotting analysis showed that a protein the same size as the recombinant Hjm protein, which also specifically reacted with the antibody, was present in the total extract from P. furiosus cells (Fig. 2B). The active fractions obtained during the purification procedure, as described above, also had a protein that reacted with the antibody (Fig. 2C). To investigate the oligomeric state of Hjm in solution, gel filtration chromatography was done (Fig. 2D). The elution profile showed that the estimated molecular weight of Hjm is 73,500, which is slightly smaller than that calculated from the deduced amino acid sequence (82, 631). This inconsistency may be explained by some interaction of Hjm with Sephade resin, because chromatographic profile showed that the elution peak of Hjm was not symmetric but had some tailing. Glycerol gradient separation experiment showed that Hjm was sedimented to the position corresponding to the molecular weight of 75,800 (Fig. 2E). These results suggest that Hjm exists as a monomeric protein in solution. Further analyses including analytical ultracentrifugation will provide more detailed information.

Cloning and Expression of the Gene Encoding the Branch Migration Helicase—Based on the reaction conditions determined from the above section, we screened for the branch migration activity from the heat-stable P. furiosus protein libraries, as described under “Experimental Procedures.” Among the 496 independent heat extracts of E. coli transformants, we found a clone producing a protein that dissolved the four-way junction DNA. The cosmid DNA was recovered from the E. coli clone, and the region containing the gene encoding the target activity was subcloned into the plasmid vector. A certain open reading frame with an ATP-binding motif (P-loop) was found after sequencing the cloned DNA. The gene for the open reading frame was cloned into the expression vector, pET21d for overexpression in E. coli. The recombinant E. coli cells carrying the resultant plasmid, pLHM100, were cultivated, and the encoded protein was successfully overproduced by isopropyl β-D-thiogalactopyranoside induction. The protein was purified to homogeneity by the three sequential chromatography steps as shown in Fig. 2A. The highly purified protein was used for the following characterization. Based on its activity to dissolve four-way junction DNA, we named the protein Hjm (Holliday Archaeal Helicase for Branched DNAs)
reaction. The RecA-mediated α-structured DNA was deproteinized by phenol extraction and then was reacted with purified Hjm. As shown in Fig. 5B, the amount of the α-structured DNA decreased, and the processed products increased with the reaction time. This result suggests that Hjm may function like bacterial RuvB in the homologous recombination process.
Hjm Homologs in Archaea and Eukarya—The deduced amino acid sequence revealed that the N-terminal region of Hjm has the seven characteristic motifs found in the helicase family (Fig. 6), and therefore, Hjm belongs to superfamily 2 in the helicase classification (30). The C-terminal region may be responsible for recognizing a specific structure of DNA or for interacting with other protein for its physiological function. The sequence of Hjm is not similar to that of the bacterial RuvB protein. Moreover, no other protein with a similar sequence to Hjm has been found in eubacteria and yeast. However, the sequence of Hjm is highly conserved in Archaea. One open reading frame sharing more than 30% amino acid sequence homology to Hjm was found in each of the genomes from both euryarchaeal and crenarchaeal organisms (Table I). Our homology search using the Hjm sequence as a query identified the human Pol\textsuperscript{H9008} and Hel308 proteins and the Drosophila Mus308 protein (Fig. 6). The \textit{mus308} gene was identified in \textit{D. melanogaster} as a gene required for resistance to DNA cross-linking reagents (31). The Hel308 protein (a helicase similar to Mus308) is a human homolog of the helicase domain of \textit{D. melanogaster} Mus308, and it has a single-stranded DNA-dependent ATPase activity with a dissociation activity for duplex DNA (32). Hel308 does not have a DNA polymerase-like sequence in the C-terminal region. Pol\textsuperscript{H9008}, with both a helicase motif and a polymerase-like sequence, is more similar to Mus308, and the POLQ gene seems to be the \textit{mus308} ortholog in the human genome (33). The DNA-dependent ATPase activity and the DNA polymerase activity of Pol\textsuperscript{H9008} have also been characterized (33). It will be very interesting to further characterize and classify these Mus308 family helicases in terms of their structures and functions.

**DISCUSSION**

We previously identified the Holliday junction resolvase, Hjc, in archaea and proposed that the formation and resolution of the Holliday junction represent a common mechanism of homologous recombination in the three domains of life (21). During our continuing efforts to identify the proteins responsible for the processing of the Holliday junction intermediate in archaea, in this study, we identified a novel helicase, which dissolves the four-way junction in an ATP-dependent manner. To isolate the target activity in the \textit{P. furiosus} cell extracts, we

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**FIG. 2.** Production and purification of Hjm protein in \textit{E. coli} cells. A, purified Hjm protein (F, 0.4 \(\mu\)g) was separated by 12% SDS-PAGE and stained by Coomassie Brilliant Blue. B, \textit{P. furiosus} cell extract (800 ng) and purified recombinant Hjm protein (1 ng) were separated by 12% SDS-PAGE, blotted onto polyvinylidene difluoride membranes, and detected by anti-Hjm sera using a chemiluminescence system. C, identification of Hjm in the cation chromatography fractions corresponding to the branch migration activity. Western blot analysis was done using the MonoS column fractions, as shown in Fig. 1B. D, gel filtration analysis. Gel filtration chromatography was performed by a SMART system equipped with a Superdex 200 PC 3.2/30. Aliquots of the fractions were subjected to 12% SDS-PAGE analysis followed by Coomassie Brilliant Blue staining. The peak positions of the marker proteins, immunoglobulin G (158 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa), eluted from the column are shown by arrows at the top. E, glycerol gradient centrifugation analysis. Hjm protein was sedimented for 24 h at 4 °C through 10–35% glycerol gradient. The standard curve was obtained by marker proteins on a parallel gradient.

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**FIG. 3.** Branch migration activity of the purified Hjm protein. Recombinant Hjm protein with the indicated concentrations was incubated with 5 \(\mu\)M of \textsuperscript{32}P-labeled HSL, 35 bp arms with a 30-bp homologous core, and the reactions were carried out at 55 °C for 30 min. \textit{T. thermophilus} RuvB and the RuvA-RuvB complex, with the indicated concentrations of each monomer protein, was used for control reactions.
used the same strategy as used for Hjc. However, we could not find the branch migration activity by using the total cell extract directly with a synthetic Holliday junction. The purification procedure to concentrate nucleic acid-binding proteins was critical to identify the target activity in this case, and the fractionation by polyethylenimine treatment followed by ammonium sulfate precipitation was very effective. Actually, the purified Hjm protein has very high affinity for DNA, and its branch migration activity was inhibited by increasing the amount of nonspecific DNA in the reaction mixture (data not shown).

It has been proposed that RuvA, RuvB, and RuvC can form a resolvasome complex and work cooperatively to process the Holliday junction (34–37). The formation of a resolvasome has also been suggested by the fact that the branch migration and junction cleavage activities in a mammalian cell-free extract fractionated together after chromatography on phosphocellulose, Butyl-Sepharose, and heparin-Sepharose columns (8). In this archaeal case, Hjm and Hjc were separated even by affinity chromatography (heparin-Sepharose). Hjc has strong affinity for heparin (21), and this interaction may be stronger than that between Hjc and Hjm if these proteins actually form the resolvasome in archaeal cells.

It seems likely that Hjm processes the homologous recombination intermediate in archaeal cells, from the result that Hjm processed the RecA-mediated H9251-structure in vitro, as shown in Fig. 5. However, it remains to be elucidated whether Hjm is the real functional counterpart of the bacterial RuvB in the Archaeal domain. Some genetic studies to analyze the phenotype of *hjm* mutants are necessary to determine the function of Hjm in cells. In terms of the early steps of homologous recombination, the homologs of the eukaryotic Mre11, Rad50, Rad51, and RPA have been found in Archaea. However, the Holliday junction resolvase, Hjc, is completely unique in Archaea, and no protein with sequence homology to Hjc has been found in Eukarya. The Holliday junction resolvases have been recognized as a very interesting issue from evolutionary aspects (24). It is possible that the proteins responsible for the processing of the Holliday junction in Archaea are totally diversified, and neither the bacterial nor eukaryotic proteins involved in this process have similarity.
A sequence homology search revealed some similarity between Hjm and eukaryotic Mus308, Hel308, and Polα/H9008 (Fig. 6).

The D. melanogaster mus308 mutant is sensitive to cross-linking agents, such as psoralen, diepoxybutane, and nitrogen mustard, and therefore, the gene product probably functions in interstrand cross-link repair, from genetic studies (31). The Mus308 protein has a family A DNA polymerase (bacterial Pol I)-like sequence at its C-terminal region. More-
shown). It remains to be determined whether the helicase activity of Hjm is related to interstrand cross-link repair in Archaea. More detailed characterizations of Hjm, in terms of its structural specificities for the DNA binding, helicase, and ATPase activities, in addition to genetic studies, will provide important information about its functions in living cells.

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