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Holliday junction-resolving enzymes from eight hyperthermophilic archaea differ in reactions with cruciform DNA

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Abstract Holliday junction-resolving enzymes have been identified in a broad variety of organisms and tissues. In this study, six new Holliday junction-cleaving enzymes (Hjcs) were obtained from hyperthermophilic crenarchaeal and euryarchaeal species, including *Pyrococcus horikoshii*, *Pyrococcus abyssi*, *Methanococcus jannaschii*, *Methanobacterium thermautotrophicum*, *Archaeoglobus fulgidus*, and *Aeropyrum pernix*. The genes were cloned and overexpressed in *Escherichia coli*, and the respective proteins were purified from crude extracts to homogeneity. For an initial characterization of the enzymatic activities, synthetic heat-stable fixed and mobile cruciform DNA substrates were used at 75°C. The Hjcs from *Pyrococcus furiosus*, *Sulfolobus solfataricus*, and the archaeal virus SIRV2 were included in the study for comparison. Despite their sequence homology, the enzymes showed marked differences in their reactions with individual cruciform DNAs. While the fixed cruciform structure was cleaved by all enzymes at only one major position, the mobile cruciform structure displayed different cleavage patterns for individual Hjcs, each with several cleavage positions. Furthermore, a strong bias for cleavage of one direction across the junction was observed with the fixed cruciform DNA for all enzymes. In contrast, the mobile cruciform DNA displayed different preferences, depending on the enzyme used.

Key words Hyperthermophilic archaea · Holliday junction · DNA metabolism · Resolvase · Recombination

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Introduction

The process of DNA recombination is essential for the generation of genetic diversity and plays an important role in specific DNA repair and replication mechanisms. It is found ubiquitously in organisms of all domains of life.

Recombination involves the physical connection of two individual DNA molecules, followed by movement of the linkage point along the parental, homologous DNA strands, known as branch migration, and, subsequently, the cleavage reaction and the release of two discrete recombinant DNA molecules.

The central structure in this process is a branched DNA molecule in which the two participating molecules are joined by the Holliday junction (Holliday 1964). There are two alternative directions of resolution of the junction, depending on the pair of opposite strands cleaved, which result in either "patch" or "splice" recombination products.

Holliday junction-resolving enzymes are structure-specific endonucleases (Kemper 1997; Lilley and White 2000). The conformation of the substrate is crucial for its recognition and final resolution. Several factors have been reported to influence the conformation of the Holliday junction in solution. These are cations (Duckett et al. 1990), the local nucleotide sequence at the junction (Altona 1996; Miick et al. 1997), and the binding of proteins (Duckett et al. 1995; Bennett and West 1995; Declais and Lilley 2000; Giraud-Panis and Lilley 1998; Pohler et al. 1996; White and Lilley 1997, 1998). The choice of conformer of the Holliday junction cleaved by the resolving enzyme leads to one of the two alternative recombination products (Li et al. 1997).

Holliday junction-resolving activities have been found in a broad variety of organisms and tissues, but the corresponding enzymes have been isolated only from *E. coli* (Dunderdale et al. 1994), bacteriophages T4 (Kemper and Garabett 1981) and T7 (de Massy et al. 1987), lambdoid prophage (Sharples et al. 1994), mitochondria of yeasts (Kleff et al. 1992; Whitby and Dixon 1997), hyperthermophilic eury- and crenarchaea (Komori et al. 1999; Kvaratskhelia and White 2000a), archaeal viruses (Birkenbihl et al. 2001), and pox viruses (Garcia et al.

2000). Besides the preferentially cleaved four-way junctions, some of these isolated enzymes also cleave other DNA structures, such as three-way junctions or substrates with single-strand loops, mismatches, or other irregular deviations from the plain duplex form (Pottmeyer and Kemper 1992; Solaro et al. 1993; Dickie et al. 1987). They all function as homodimers, require divalent cations for cleavage, and resolve the Holliday junction by introducing pairs of nicks symmetrically located across the junction. All Holliday junction-resolving enzymes bind their substrate in a sequence-independent mode, but some show preferences for certain sequences for cleavage (White and Lilley 1996; Shah et al. 1994).

Despite having equal enzymatic activities, the isolated Holliday junction-resolving enzymes have evolved from different structural folds (Aravind et al. 2000; Lilley and White 2000). While Holliday junction-cleaving enzymes (Hjcs) from archaea and their viruses show high sequence similarity among themselves, they do not exhibit significant homology to their nonarchaeal counterparts. Homology and mutational studies of the archaeal Hjcs indicate a phylogenetic relationship to type II restriction endonucleases (Daiyasu et al. 2000; Kvaratskhelia et al. 2000). This was confirmed by the recently resolved crystal structure of *Pyrococcus furiosus* (Pfu)-Hjc, which showed structural similarities to the restriction enzymes *Eco*RV, *Bgl*I, and *Fok*I, but not to any other Holliday junction-resolving enzymes with known structure (Nishino et al. 2001).

Except in the case of *Thermoplasma acidophilum*, putative genes for Hjcs are present in every sequenced archaeal genome. So far, only Pfu-Hjc, *Sulfolobus solfataricus* (Sso)-Hjc, and Hjcs from viruses infecting *S. islandicus* (Sis) have been functionally analyzed (Komori et al. 2000b; Kvaratskhelia and White 2000a; Birkenbihl et al. 2001).

In this study, six new putative Hjcs were obtained from hyperthermophilic cren- and euryarchaeal species, and their predicted enzymatic activities were verified. The genes were cloned and overexpressed in *E. coli*, and the respective proteins were purified. For an initial characterization of the enzymatic activities, heat-stable fixed and mobile synthetic cruciform DNA substrates were used. Pfu-Hjc, Sso-Hjc, and the viral SIRV2-Hjcs were included in the study for comparison. Surprisingly, the homologous enzymes showed marked differences in their reactions with individual cruciform DNAs.

Materials and methods

Cloning of archaeal *hjc* genes

The accession numbers of the *hjc* genes are as follows: *Methanobacterium thermautotrophicum* (Mth), gb:AE000893; *Archaeoglobus fulgidus* (Afu), gb:AE000967; *Pyrococcus horikoshii* (Pho), gb:AP000006; *Pyrococcus abyssi* (Pab), gb:AJ248285; Pfu, gb:AB023635; *Methanococcus jannaschii* (Mja), sp:Y497_METJA; *Aeropyrum pernix* (Ape), gb:AP000059; and Sso, gb:Y18930.

The genes for the archaeal Hjcs were amplified directly from genomic DNA by using PCR analysis. With the exception of Sis DNA, which was prepared from cells obtained from the research group of W. Zillig (MPI Biochemie, Martinsried, Germany), genomic DNA was prepared from cells purchased from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). All *hjc* genes were amplified by PCR by using the DNA-oligonucleotide 5′-TAGACCGCGGCAT-3′ followed by differing numbers of nucleotides (nt) from the 5′ sequence of the respective genes as the sense primer. These were for Pfu, 24 nt; Pho, 25 nt; Pab, 24 nt; Afu, 24 nt; Ape, 24 nt; Mja, 28 nt; Mth, 25 nt; and Sso/Sis, 26 nt. The antisense primers consisted of differing numbers of nucleotides from the 3′ sequence of the respective genes following the DNA-oligonucleotide 5′-CGGGGTACC-3′. These were for Pfu, 26 nt; Pab, 25 nt; Afu, 25 nt; Ape, 25 nt; Mja, 30 nt; Mth, 24 nt; and Sso/Sis, 28 nt. In the case of Pho, the antisense primer was 5′-ATACGGGAGCTC-3′ followed by the sequence of the last 25 nt of the *hjc* gene. By the use of these primers, the resulting PCR products contained recognition sites for restriction enzymes *Nde*I and *Sac*II, located 5′ of the *hjc* gene sequence, and *Kpn*I, which is 3′ of the *hjc* gene sequence. The PCR product obtained by using Pho DNA as a template contained a *Sac*I site instead of the *Kpn*I site, because there is a *Kpn*I site inside the Pho *hjc* gene. The PCR analyses were performed by using 10 nmol primers and 0.5 U proofreading *Pwo* DNA polymerase under conditions recommended by the supplier (Boehringer Mannheim, Germany), with 30 reaction cycles (1 min 94°C, 2 min 58°C, 2 min 72°C).

The *Nde*I/*Kpn*I (Pho: *Nde*I/*Sac*I) digested PCR products were ligated into a vector pET11a (Studier et al. 1990) derivative called pRB371. The resulting plasmids were named as follows: Pfu, pKN101; Pho, pKN102; Afu, pKN103; Ape, pKN104; Mja, pKN105; Sso, pKN106; Mth, pKN107; Sis, pKN108; Pab, pKN110. The cloned genes were sequenced from both ends to verify the sequences predicted from the database. In the case of the PCR analysis using Sis genomic DNA as a template, the resulting product had the same sequence as the Sso *hjc* gene, so it was not analyzed individually.

Expression and purification of recombinant archaeal Hjcs

For expression, the plasmids pKN101–pKN108 and pKN110 were transformed into *E. coli* strain BL21 (DE3) Codon Plus RIL (Stratagene, La Jolla, CA, USA), which contains a plasmid carrying extra genes for tRNAs, which are rare in *E. coli*. The cells were grown at 37°C in 500 ml LB medium with 100 µg/ml ampicillin and 87 µg/ml chloramphenicol to an OD_{600} of 0.8. After induction with 1 mM isopropyl thiogalactoside (IPTG), final concentration, the cells were grown for another 2 h. The cells were harvested by centrifugation and resuspended in 10 ml lysis buffer [50 mM Tris HCl, pH 8; 0.1 mM EDTA; 0.5 mM dithiothreitol (DTT); 10% glycerol; 1 M KCl; 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The cells were disrupted by sonication and,

after another 10 ml of lysis buffer was added, the lysates were cleared by centrifugation at 30,000 *g* for 30 min. The following purification procedure is a modified version of the protocol by Komori et al. (1999). The cleared lysate was mixed with polyethyleneimine (pH 8) to a final concentration of 0.1% and centrifuged at 30,000 *g* for 30 min. The resulting supernatant was dialyzed against buffer A (50 mM Tris HCl, pH 8; 0.1 mM EDTA; 0.5 mM DTT; 10% glycerol), followed by protein precipitation with ammonium sulfate (80% saturation). The precipitate was resuspended in and dialyzed against buffer B (0.1 M potassium phosphate, pH 6.8; 10 mM 2-mercaptoethanol; 10% glycerol) and then heated to 80°C for 30 min. The supernatant from centrifugation of the heat-treated fraction was applied onto a 5-ml hydroxyapatite column (Bio-Rad, München, Germany), and was developed with a 0.1–1 M potassium phosphate gradient in buffer B. Fractions containing >90% Hjc were eluted at 0.2 M (Sso, Mth), 0.4–0.6 M (Pfu, Pho, Pab, Afu, Ape), or 0.7 M potassium phosphate (Mja). The corresponding fractions were dialyzed against and stored in buffer BK (10 mM Tris HCl, pH 8; 150 mM KCl; 10 mM 2-mercaptoethanol; 10% glycerol) at –20°C.

DNA substrates

CFKla01 is a cruciform DNA with a homologous core of 10 nt allowing strand migration over 10 bp. With a GC content of 66% and arm lengths of 19–24 bp, it was designed for greater stability at high temperatures. Each arm contained a unique restriction site for one of the heat-stable restriction enzymes *Bsi*HKAI, *Tth*111I, *Taq*I, and *Bsr*I. These enzymes were used to test the structural integrity of the substrates at 65°C. In addition, each arm contained a unique restriction site for one of the enzymes *Bam*HI, *Xba*I, *Eco*RI, and *Pst*I, located closer to the center of the structure, and a central *Bsi*WI site at the junction, which were used to create size markers for reference. Together with DNA ladders derived from each strand, the restriction fragments were used to map the cleavage sites generated by the resolving enzymes at the nucleotide level.

A second structure, CFKla11, is a closely related derivative of CFKla01 with an immobile junction. It was made by exchanging the central 5 bp from two neighboring arms, thus disrupting the homology core and removing the central *Bsi*WI sites.

Eight oligonucleotides were used for assembling the two substrates. The mobile four-way junction CFKla01 (the homology core sequences allowing branch migration are in italics) was assembled with oligo 01, 46 nt, GCTCGCGTGCACGGATCC*GGCGTACGCC*ACTGCAGCCAGTCGGATG, restriction sites for *Bsi*HKAI, *Bam*HI, *Bsi*WI, *Pst*I, and *Bsr*I are underlined; oligo 02, 47 nt, AGAGGCGACGCGGTCTAGAG*GGCGT ACGCC*GGATCCGTGCACGCGAG, sites for *Tth*111I, *Xba*I, *Bsi*WI, *Bam*HI, and *Bsi*HKAI are underlined; oligo 03, 43 nt, ATGCCTCGAATTC A*GGCGT ACGCC*CTCTAGACCGCGTCGCCTC, sites for *Taq*I, *Eco*RI, *Bsi*WI, *Xba*I, and *Tth*111I are underlined; and oligo 04, 44 nt, CCATCCGACTGGCTGCAGT*GGCGT ACGCC*TTGAATTCGAGGCATT, sites for *Bsr*I, *Pst*I, *BsiW*I, *Eco*RI, and *Taq*I are underlined. The fixed four-way junction CFKla11 was assembled with oligos 11–14. These were the same as oligos 01–04 except for the homology core sequences, which were replaced by the following sequences: oligo 11, GGCGTACGCC; oligo 12, GGCGTTGCGG; oligo 13, identical to oligo 03; oligo 14, CCGACACGCC.

The oligos were (5′-^{32}P)-end labeled with T4 polynucleotide kinase and assembled by hybridization in TBE (80 mM Tris, 80 mM borate, 2 mM EDTA) containing 10 mM $MgCl_2$. After purification over a native polyacrylamide (PAA) gel, the substrates were eluted in TE buffer (10 mM Tris, pH 8.0; 0.1 mM EDTA) and used in the assays without further treatment.

Endonuclease assays

The assay conditions given for Pfu-Hjc (Komori et al. 1999) were applied to all Hjcs used. No attempts were made to optimize the individual requirements of each enzyme for pH, salt, or divalent cations. If not indicated otherwise, cleavage assays were performed as follows: 10 nM ^{32}P-5′-radioactively labeled cruciform DNA was incubated with 30 nM Hjc protein in reaction buffer (20 mM Tris pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) at 75°C for 30 min. The 10-µl reaction was stopped by the addition of 5 µl of MG-stop buffer (90% deionized formamide, 0.1% bromphenol blue, 0.1% xylene cyanol in TBE). The mixture was then heated for 1 min at 95°C and run on a 12.5% denaturing PAA gel in TBE. Gels were analyzed by phosphor imaging.

Results

Identification and sequence homology of archaeal Hjcs

The putative archaeal Holliday junction-cleaving enzymes (Hjcs) were identified by using the Pfu-Hjc sequence as a template for a BLAST (Altschul et al. 1990) database search. Two of the seven top-scoring sequences, Sso-Hjc and Ape-Hjc, belonged to crenarchaeal species, while five were of euryarchaeal origin. The latter included two pyrococcal species. The total number of amino acids of these Hjcs varied from 121 to 143, giving calculated molecular weights from 13.4 to 16.0 kDa. The calculated pI values of all Hjcs were basic in the range 9.2–10.8.

The multiple alignment shown in Fig. 1A includes the two viral Hjcs, SIRV1-Hjc and SIRV2-Hjc, which have been described recently (Birkenbihl et al. 2001). The cellular Hjcs showed a high sequence similarity with amino acid identities between 35% and 64% to Pfu-Hjc. The lowest similarity in this compilation was shown by the viral SIRV-Hjcs with 30% and 32% identity. The highest similarity to Pfu-Hjc was shown by the pyrococcal Pho-Hjc and Pab-Hjc with 64% and 62% identity, respectively. The N-terminal halves of the enzymes were highly similar, harboring all completely

conserved residues. The C-terminal parts displayed only low similarity and showed various gaps in the alignment of the sequences.

There were ten completely conserved amino acid residues in the sequences of all Hjcs (Fig. 1). Six of them comprised the motif G-X_3-E-X_{10}-G-X_{11-18}-D-X_{12}-E-X-K, defining the archaeal Holliday junction resolvase (AHJR) family, which belongs to superfamily I of the DNases of the nuclease fold (Aravind et al. 2000). This motif contains the submotif D-X_{6-30}-D/E-X-K, which is typical for type II restriction endonucleases (Daiyasu et al. 2000), and which has been shown to be directly involved in binding Mg^{2+}, necessary for nuclease activity (Kostrewa and Winkler 1995). Some of the conserved residues, including the AHJR motif for Pfu-Hjc and the submotif for Sso-Hjc, have been shown by mutational analyses to be essential for cleavage (Komori et al. 2000a; Kvaratskhelia et al. 2000).

Not all of the residues shown to be essential in Pfu-Hjc were conserved among the other Hjcs. The change of phenylalanine to alanine at position 72 in Pfu-Hjc inactivated the enzyme by destroying its ability to dimerize. Interestingly, the corresponding amino acid residue in Ape-Hjc is an alanine (A81), which did not affect the ability of this Hjc to dimerize, as confirmed by cross-linking experiments (data not shown). Furthermore, the Sso-Hjc sequence, which shows a serine (S82) in the position of the Pfu-Hjc F72, was also dimerization proficient. Two other residues (F68 and K81) that have been shown to be essential for Pfu-Hjc activity differ from the corresponding residues in the aligned Ape-Hjc sequence (W77 and R90), but these are conservative amino acid exchanges.

According to the proposed model of the Hjc–Holliday junction complex (Nishino et al. 2001), basic residues located in the N-terminal part of the α_1-helix, in the two loops between sections β_1 and β_2 and β_3 and β_4, are in direct contact with the DNA substrate (Fig. 1). Differences in the amino acid sequence in these regions can be expected to have an effect on Hjc–substrate interactions. Notably, a seven-amino-acid deletion found in the pyrococcal Hjcs and Mja-Hjc was located in the loop between sections β_1 and β_2. Furthermore, the Afu-Hjc did not contain any basic residues in this loop, and all Hjcs showed differences in the distribution and frequency of basic residues in the α_1-helix. These observations lead us to initiate an investigation of the specific cleavage behavior of the available Hjcs.

Cloning and purification of homologous Hjcs

The *hjc* genes were amplified by PCR directly from genomic DNA, cloned into an expression vector, and overexpressed in *E. coli*. After induction of expression and 2 h of further growth, a new protein band became visible in crude cell lysates on an SDS-PAGE gel. During the purification procedure described in the Materials and methods section, the protein corresponding to this new band was isolated. Yields of Hjc proteins ranged from 0.5 to 5 mg from 500-ml expression cultures.

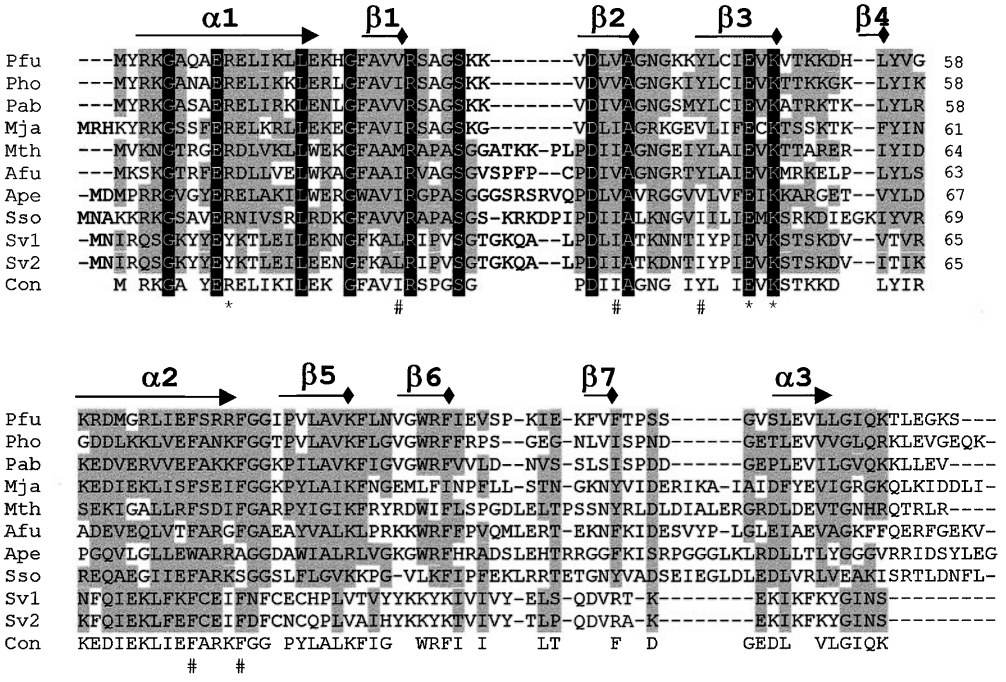

Fig. 1. Multiple sequence alignment of the eight archaeal Hjcs by the program Vector NTI using the clustal W algorithm (Thompson et al. 1994). Completely conserved residues are printed *white on black*, similar residues are printed *black on gray*. The *arrows* on top of the alignment indicate the positions of α-helices and β-strands as seen in the crystal structure of Pfu-Hjc; *asterisks* indicate essential residues for endonuclease activity of Pfu-Hjc; *hash marks* indicate essential residues for dimerization for Pfu-Hjc. Abbreviations used: *Pfu*, *Pyrococcus furiosus*; *Pho*, *Pyrococcus horikoshii*; *Pab*, *Pyrococcus abyssi*; *Mja*, *Methanococcus jannaschii*; *Mth*, *Methanobacterium thermautotrophicum*; *Afu*, *Archaeoglobus fulgidus*; *Ape*, *Aeropyrum pernix*; *Sso*, *Sulfolobus solfataricus*; *Sv1*, SIRV1; *Sv2*, SIRV2; *Con*, consensus sequence

None of the purified archaeal Hjcs showed a band on the SDS-PAGE gel corresponding exactly to its calculated molecular weight (Fig. 2). All proteins migrated more slowly than expected, although to different extents. This aberrant mobility effect was previously observed for purified Pfu- and Sso-Hjcs (Komori et al. 1999; Kvaratskhelia and White 2000a). The high polarities of the proteins might explain this effect, as described for other proteins (Lee et al. 1991; Query et al. 1989). The purity of the isolated Hjcs was generally higher than 95%. In the case of Mth-Hjc, two additional faint bands were visible on the gel (Fig. 2).

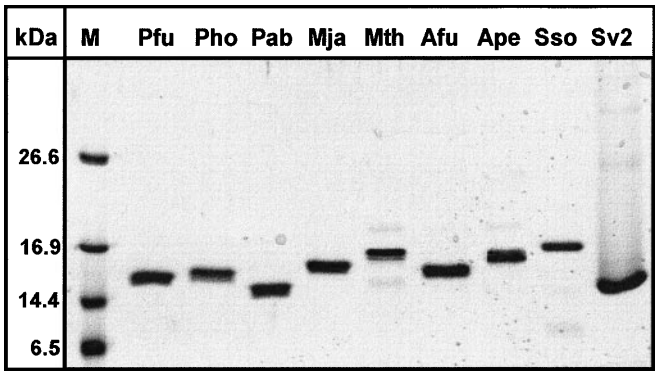

Fig. 2. SDS-PAGE (15%) analysis of purified recombinant archaeal Hjcs (1 µg per lane). The gel was stained with Coomassie brilliant blue. *M* indicates a lane with marker proteins of defined molecular weights

Cleavage activities of the Hjcs

Holliday junction-resolving enzymes cleave the Holliday junction in a symmetrical mode by introducing two nicks in opposite strands across the junction. The positions and efficiencies of cleavages in a given substrate differ among Hjcs according to their individual substrate specificity. Evidence has been presented that the local sequence at the junction and the spatial conformation of the cruciform DNA can influence the cleavage behavior of a given Holliday junction-resolving enzyme.

The cleavage specificities of the Hjcs were determined by using two related synthetic cruciform DNAs: CFKla01, which allowed branch migration over ten nucleotides, and CFKla11, with a fixed junction. These substrates were designed for increased stability at temperatures of up to 80°C to allow reaction conditions close to the natural environment of hyperthermophiles.

All archaeal Hjcs cleaved both substrates through the introduction of symmetrical nicks located in opposite

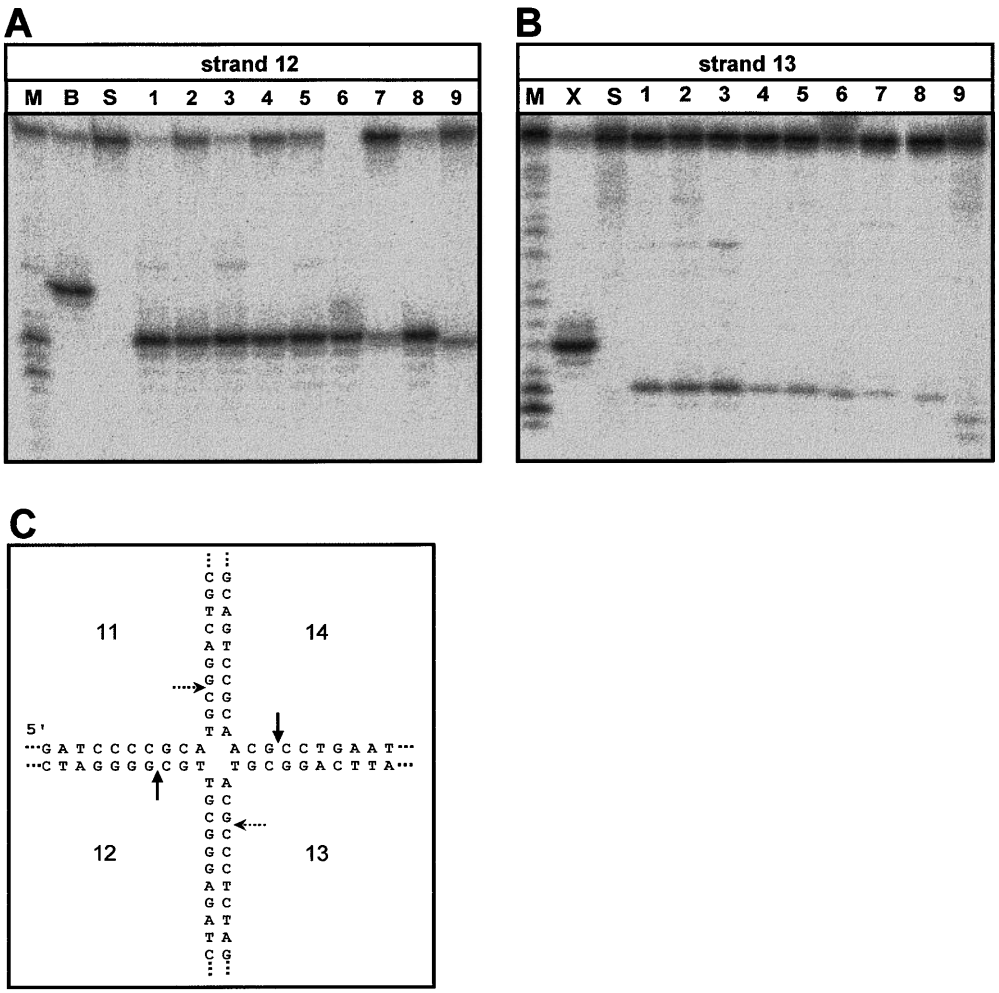

Fig. 3A–C. Cleavage of fixed cruciform DNA CFKla11 by archaeal Hjcs. **A, B** A 10-nM solution of CFKla11 was incubated with 30 nM of each recombinant archaeal Hjc as described in Materials and methods. The reaction products were separated by electrophoresis on a denaturing 10% polyacrylamide gel and visualized by phosphor imaging. The substrate was (5′-^{32}P)-labeled on either strand 12 (**A**) or 13 (**B**). *M* indicates marker lanes containing DNaseI digested substrate; *S* indicates lanes with substrate only; *B* and *X* indicate lanes with digestions of the substrate with *Bam*HI and *Xba*I, respectively, providing reaction products of a defined length. *Numbered lanes* indicate reaction products from incubations of the substrate with the recombinant archaeal Hjcs: *1*, Pfu-Hjc; *2*, Pho-Hjc; *3*, Pab-Hjc; *4*, Mth-Hjc; *5*, Ape-Hjc; *6*, Mja-Hjc; *7*, Afu-Hjc; *8*, Sso-Hjc; *9*, SIRV2-Hjc. **C** Nucleotide sequence of the central region of CFKla11 with cleavage positions indicated by *arrows*. All Hjcs cleave the substrate at the same positions. *Solid arrows* denote strong cleavage sites, *dashed arrows* denote weak cleavage sites (cleavage efficiencies <10%)

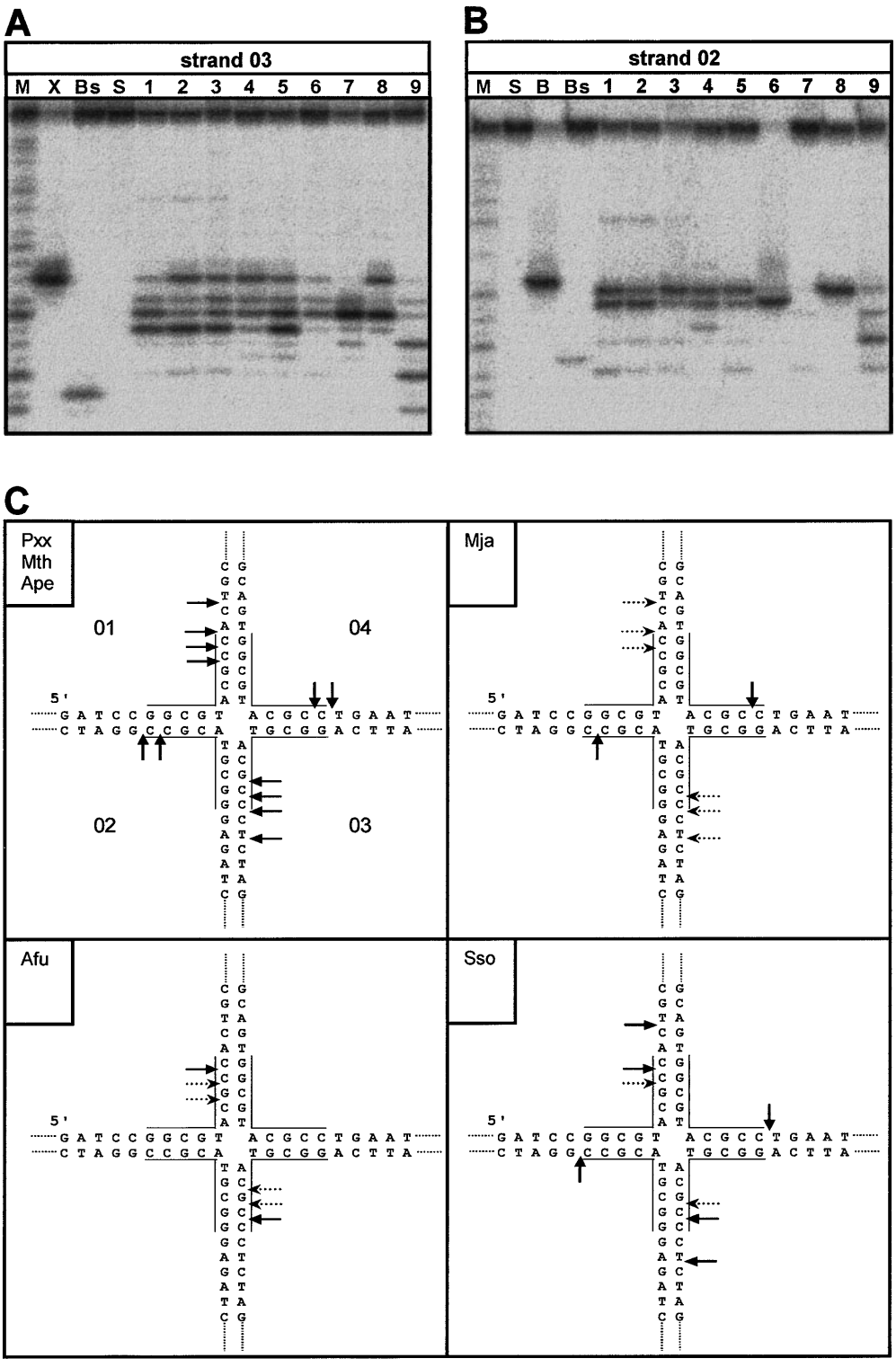

Fig. 4A–C. Cleavage of mobile cruciform DNA CFKla01 by archaeal Hjcs. **A, B** A 10-nM solution of CFKla01 was incubated with 30 nM of each recombinant archaeal Hjc as described in Materials and methods. The reaction products were separated by electrophoresis on a denaturing 10% polyacrylamide gel and visualized by phosphor imaging. The substrate was (5′-^{32}P)-labeled on either strand 03 (**A**), or strand 02 (**B**). *M* indicates the marker lanes with DNaseI digested substrate; *S* indicates lanes with substrate only; *B*, *Bs*, and *X* indicate lanes with digestions of the substrate with *Bam*HI, *Bsi*WI, and *Xba*I, respectively, providing reaction products of defined length. *Numbered lanes* indicate reaction products from incubations of the substrate with the recombinant archaeal Hjcs: *1*, Pfu-Hjc; *2*, Pho-Hjc; *3*, Pab-Hjc; *4*, Mth-Hjc; *5*, Ape-Hjc; *6*, Mja-Hjc; *7*, Afu-Hjc; *8*, Sso-Hjc; *9*, SIRV2-Hjc. **C** Nucleotide sequence of the central region of CFKla01 with cleavage positions indicated by *arrows*. The mobile homologous region is marked by *thin lines*. Four different cleavage patterns can be distinguished for the eight Hjcs, which are represented in the four schemes. *Solid arrows* denote major cleavage sites, *dashed arrows* denote minor cleavage sites (<10% cleavage efficiencies)

strands 3′ of the junction. In both substrates, this resulted in identical cleavage patterns for opposite strands. Therefore, only one strand of each pair is shown in Figs. 3 and 4.

The pairs of opposite strands are 01/03 and 02/04 in CFKla01 and 11/13 and 12/14 in CFKla11.

All eight cellular Hjcs cleaved CFKla11 at identical positions, three nucleotides 3′ of the junction (Fig. 3). The same cleavage position has recently been reported for Pfu-Hjc and Sso-Hjc with other fixed substrates (Komori et al. 1999; Kvaratskhelia and White 2000a). Interestingly, the two viral Hjcs SIRV1-Hjc and SIRV2-Hjc cleave the same strands only two nucleotides 3′ from the junction (Birkenbihl et al. 2001) (lane 9 in Fig. 3A and B).

All Hjcs showed a strong bias for cleavage axis 12/14 (Fig. 3A) with more than 80% of the substrate being cleaved, while only less than 10% along axis 11/13 was cleaved (Fig. 3B). The same bias in cleavage was found with viral SIRV-Hjcs (lane 9 in Fig. 3A and B).

Because all strands at the junction had the identical sequence, this result strongly suggests that the structural constraint of the construct, rather than its immediate sequence, was responsible for the biased cleavage. Structural constraint has been shown to be the reason for cleavage of only continuous strands of a fixed junction by Sso-Hje (Kvaratskhelia and White 2000b), another Holliday junction-cleavage activity from Sso. In the same study, Sso-Hjc was shown to lack any cleavage bias, cleaving both continuous and exchanging strands with equal efficiencies. This is in contrast to our results for Sso-Hjc using the fixed junction CFKla11 (Fig. 3A and B, lane 8).

When the substrate CFKla01 with a mobile junction was used, the cleavage patterns were remarkably different (Fig. 4). With the exception of Afu-Hjc (Fig. 4A and B, lane 7) and Mja-Hjc (Fig. 4A and B, lane 6), all Hjcs cleaved along both axes with equal efficiencies. Afu-Hjc had a strong bias for the 01/03 axis, while Mja-Hjc cleaved along the 02/04 axis more efficiently. The cleavage positions are summarized in Fig. 4C. As indicated in the figure, all cleavages along the 01/03 axis were localized within a range of six nucleotides, beginning two nucleotides 3′ from the center of the homology. The pyrococcal (Pxx-), Mth-, and Ape-Hjc cleaved at the same four positions, although each with a different efficiency (solid arrows in Fig. 4C, upper left panel). Mja-Hjc cleaved weakly at three positions (dashed arrows in Fig. 4C, upper right panel). Afu-Hjc cleaved at three positions with a strong preference for the outermost position (Fig. 4C, lower left panel), while Sso-Hjc cleaved at three positions with a strong preference for the two outer positions (Fig. 4C, lower right panel).

The grouping of the Hjcs with respect to the cleavage positions along the 02/04 axis is the same as for the 01/03 axis. Here, the cleavage positions occurred in a range of two nucleotides, at positions four and five 3′ of the mobile junction (Fig. 4B). Pxx-, Mth-, and Ape-Hjc cleaved at both positions but with different efficiencies (Fig. 4C, upper left panel). Mja-Hjc cleaved with high efficiency at one position, four nucleotides 3′ of the junction (Fig. 4C, upper right panel). Afu-Hjc did not visibly cleave along the 02/04 axis (Fig. 4B, lane 7), and Sso-Hjc cleaved at one position, five nucleotides 3′ of the junction (Fig. 4C, lower right panel).

The cleavage positions of the viral SIRV2-Hjcs were in all instances remarkably different from those of the cellular Hjcs (Fig. 4A and B, lane 9).

Discussion

Following a database search for Pfu-Hjc homologues, six new open reading frames (ORF) were identified in hyperthermophilic archaea. The ORFs were cloned and expressed in *E. coli*, and the purified proteins were functionally examined. The already known Pfu-Hjc, Sso-Hjc, and viral SIRV2-Hjc were included in the analyses. We show here that the six new proteins are indeed junction-cleaving enzymes that specifically react with synthetic cruciform DNAs. Following the conserved mode of junction resolution known from all resolvases, they introduced symmetrically positioned nicks in opposite strands across the junction.

All cellular Hjcs cleaved the four strands of the immobile cruciform substrate CFKla11 at the same positions located three nucleotides 3′ of the branch point (Fig. 3). Furthermore, all enzymes showed strong preference for the same opposite pair of strands, 12 and 14, marking one cleavage axis. The result was markedly different when the mobile cruciform substrate CFKla01 containing a 10-bp branch migratable junction was used. Depending on the Hjc, distinctive patterns of cleavage positions and efficiencies were observed (Fig. 4), with cleavage positions in a range of two or six nucleotides for cleavage axes 02/04 or 01/03, respectively. Again, preferences for introducing nicks in strands along one cleavage axis were observed, but they varied among the Hjcs.

Since the branch point in CFKla01 is mobile within 10 bp, the substrate preparation potentially contains a mixture of up to ten different substrates, each unique in its combination of junction-flanking nucleotides and arm lengths that change with each step of branch migration. In solution and in the presence of divalent cations, cruciform DNAs exist in a characteristic stacked-X structure (reviewed in Lilley 2000). Any given cruciform DNA can in principle adopt two alternative conformations, which differ in how stacked DNA duplexes are combined. The immediate nucleotide sequence at the junction mainly determines the ratio of these conformers. Thus, CFKla01 contains a typical mixture of up to 40 distinct substrates, of which 20 may be realized if one counts only those conformers with antiparallel-oriented stacking arms (Lilley and White 2001) that differ in the sequence at the junction and in the stacking partners of the arms. The choice of conformer cleaved determines the resolution along the one or the other axis of the Holliday junction, and, consequently, the ratio of "patch" to "splice" recombinants among progeny (Cromie and Leach 2000). The Hjcs will select from the broad collection of structures provided by a cruciform substrate with a mobile junction whatever fits their individual preferences best.

Since no preferentially cleaved nucleotide sequences could be identified with the substrates used, the bias for one cleavage axis over the other, as seen for all Hjcs with the fixed substrate (Fig. 3C) and most strikingly for Afu-Hjc and Mja-Hjc (Fig. 4C) with the mobile substrate, can be interpreted as a structure-related specificity of the Hjcs.

Since the branch point in CFKla01 is mobile, all nicks could have been introduced at a distance three nucleotides 3′ from an individual branch point, as was observed with the fixed cruciform CFKla11. This would agree with the assumption that active Hjc dimers have a fixed distance between catalytic centers.

Concerning the cleavage patterns with CFKla01, the three pyrococcal enzymes can be grouped together with

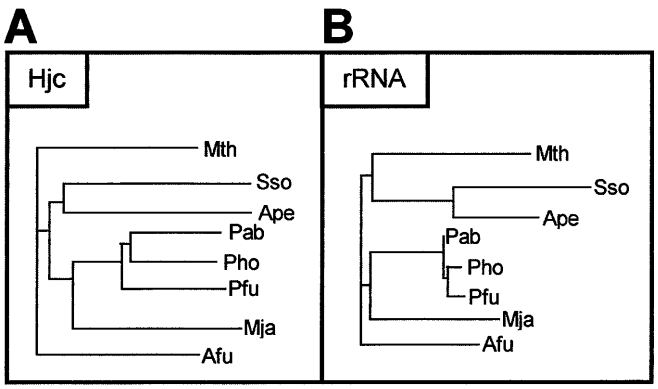

Fig. 5. A Phylogenetic tree of the archaeal Hjcs based on the amino acid sequences. **B** Phylogenetic tree of the archaeal 16S rRNAs, calculated and drawn with Vector NTI software by using the neighbor-joining method (Saitou and Nei 1987)

Mth-Hjc and Ape-Hjc, while Mja-Hjc, Afu-Hjc, Sso-Hjc, and the SIRV2-Hjc produced markedly different patterns. Interestingly, the viral SIRV2-Hjc, the Hjc with the most divergent sequence from the other Hjcs, also showed the greatest differences in the cleavage pattern compared to the cellular enzymes.

In order to see whether this grouping could be correlated with the divergence of the Hjcs, a phylogenetic tree of the Hjc amino acid sequences was constructed by using the computer program Vector NTI (InforMax, North Bethesda, MD, USA) (Fig. 5A). The phylogenetic analysis showed that the three pyrococcal Hjcs were more closely related to each other than to the remaining Hjcs. The next closest relative to the pyrococcal Hjcs was Mja-Hjc. This relationship is emphasized by a seven-amino-acid deletion in the conserved N-terminal portion that was shared only by these Hjcs (Fig. 1A). The crenarchaeal Sso- and Ape-Hjcs formed a group distinct from the other enzymes, being of euryarchaeal origin. The Afu- and Mth-Hjcs were not closely connected to any of these groups. The relationships among the Hjcs shown in this phylogenetic tree correlate well with the general phylogenetic relationship of these archaeal species, according to their 16S rRNA sequences (Fig. 5B). The grouping of the enzymes by their cleavage patterns is therefore not reflected by these phylogenetic trees. For example, the phylogenetically distantly related Mth-Hjc and Pfu-Hjc showed nearly identical cleavage patterns, whereas the more closely related Pfu-Hjc and Mja-Hjc differed markedly. One should, however, keep in mind that the specificities of the Hjcs were examined under in vitro conditions with two defined synthetic substrates, and the in vivo functions may correlate more closely with their evolutionary relationships.

The question remains, which differences in amino acid sequences between the homologous enzymes are responsible for the obvious differences in substrate specificity among the Hjcs? The recently resolved crystal structure of Pfu-Hjc might provide some clues (Nishino et al. 2001). The proposed binding mode of Pfu-Hjc to the cruciform DNA predicts certain regions of the enzyme containing basic amino acid residues to be in direct contact with the DNA. Comparing corresponding regions in the other Hjcs, differences in frequency and distribution of basic residues can be observed. Mutational analysis of these regions in the Hjcs and subsequent testing of the mutants for specificity changes may lead to an answer.

Widespread, homologous enzymes usually play key roles in cellular processes, having evolved early and under strong selective pressure, because of their importance for the cell. A good example is the ubiquitous strand-transfer protein RecA, which is essential for pairing of molecules in recombination and has homologues in all domains of life. The high homology of the Hjcs and their presence in almost all archaeal species sequenced so far suggests a conserved function, which must be revealed by further genetic studies. In general, the Hjcs are expected to be involved in processes requiring cleavage of branched DNAs as they occur during recombination, repair, or replication.

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