Multiple Holliday junction resolving enzyme activities in the Crenarchaeota and Euryarchaeota

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Abstract Holliday junction resolving enzymes are required by all life forms that catalyse homologous recombination, including all cellular organisms and many bacterial and eukaryotic viruses. Here we report the identification of three distinct Holliday junction resolving enzyme activities present in two highly divergent archaeal species. Both *Sulfolobus* and *Pyrococcus* share the Hjc activity, and in addition possess unique secondary activities (Hje and Hjr). We propose by analogy with the two other domains of life that the latter enzymes are viral in origin, suggesting the widespread existence of archaeal viruses that rely on homologous recombination as part of their life cycle. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Homologous recombination; Holliday junction; Resolving enzyme; Nuclease; Archaeon

1. Introduction

Holliday junction resolving enzymes are a ubiquitous class of nucleases that have evolved to recognise the structure of four way DNA junctions (reviewed in [1,2]). Holliday junctions are created during the process of homologous recombination, where they link homologous DNA duplexes, with the junction centre formed at the point of strand exchange between the two duplex species [3]. Branch migration of junctions, which can be either spontaneous or enzyme catalysed, increases the extent of strand exchange between homologous DNA duplexes. The process of homologous recombination is completed by junction resolving enzymes, which introduce paired nicks in opposing strands of the junction, resulting in the generation of recombinant DNA duplex products. The importance of this pathway is emphasised by the observation that the strand exchange protein RecA/Rad51 is the only DNA repair protein conserved in all genomes sequenced to date [4], and by the identification of Holliday junction resolving enzymes in many cellular and viral organisms, including eubacteria and their phage, eucarya and their viruses, and most recently in the archaea.

Holliday junction resolving enzymes are all dimeric, magnesium dependent nucleases with strong structural specificity (Table 1). There is a fundamental division between the enzymes that are members of the integrase superfamily and those of the nuclease superfamily [5]. The former includes the eubacterial enzyme RuvC, mitochondrial enzyme Cce1 and pox viral resolving enzyme [6], which all exhibit a strong degree of nucleotide sequence dependence for activity. The nuclease superfamily includes the bacteriophage enzyme T7 endonuclease I and the archaeal enzyme Hjc, which show no sequence dependence. The mechanisms by which the resolving enzymes recognise, manipulate and cleave four way DNA junctions have been studied using a wide variety of biophysical methods including comparative gel electrophoresis, 2-aminopurine fluorescence [7], isothermal titration calorimetry [8] and pre-steady state kinetics [9]. In spite of this effort, and the solution of crystal structures for RuvC [10], T4 endonuclease VII [11] and T7 endonuclease I [12], some key questions regarding the molecular mechanisms of substrate recognition and cleavage remain unanswered [5].

The archaea constitute a third domain of life, and archaeal information processing pathways have many similarities to the equivalent pathways in eukaryotes [13]. Increasingly, archaeal transcription, DNA replication and repair processes are regarded as valuable, simplified models for the much more complex eukaryotic processes. In the pathway of homologous recombination, the archaeal strand exchange protein RadA is significantly more similar to Rad51 than to the eubacterial RecA protein [14], suggesting that the archaeal recombination machinery might resemble that in eukaryotes. Recent studies of archaeal Holliday junction resolving enzymes resulted in the identification of the Hjc protein in the euryarchaeon *Pyrococcus furiosus* [15] and crenarchaeon *Sulfolobus solfataricus* [16,17]. Hjc is conserved in all archaea sequenced to date, and therefore probably constitutes the cellular archaeal enzyme, analogous to RuvC in eubacteria. However, we have previously demonstrated the presence of a second Holliday junction resolving enzyme, named Hje, in *Sulfolobus*. Although this enzyme has not yet been identified it has been characterised in some detail, and displays some significantly different characteristics from Hjc, notably in its unique specificity for cleavage of the continuous strands of stacked-X junctions [18].

In this paper we report the identification of a second Holliday junction resolving activity (named Hjr) in extracts of *P. furiosus*, and a comparison of the substrate specificity of the four enzymes found in *Pyrococcus* and *Sulfolobus*. We demonstrate that the Hjc enzymes from the two organisms have very similar activities, but that the characteristics of the Hje and Hjr activities differ from one another significantly. We postulate that the secondary activities in these two highly divergent archaeal species may be virally encoded, as has

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Table 1
Summary of known Holliday junction resolving enzymes

Superfamily	Resolving enzyme	Organism	Sequence dependence (recognition sequence)
Nuclease	Hjc	Archaeal cellular	No
	T7 endonuclease I	Bacteriophage T7	No
Integrase	RuvC	Eubacterial cellular	Yes (5′-TT/)
	Cce1	Fungal mitochondria	Yes (5′-CT/)
	A22	Pox viruses	Yes?
Unknown	RusA	Lambdoid phage	Yes (5′-/GG)
	T4 endonuclease VII	Bacteriophage T4	No
	Hje/Hjr?	Archaeal viral?	No

been demonstrated for the bacteriophage and pox viral enzymes from the two other domains of life.

2. Materials and methods

2.1. Partial purification of archaeal resolving enzymes

The *S. solfataricus* P2 and *P. furiosus* biomass was supplied by the Centre for Extremophile Research, Porton Down, UK. Cell lysis, centrifugation and chromatography steps were carried out at 4°C. 50 g cells were thawed in 150 ml lysis buffer and immediately sonicated for 5×1 min with cooling. The lysate was centrifuged at 40 000×*g* for 30 min. The supernatant was diluted four-fold with buffer A and applied to an SP-Sepharose High Performance 26/10 column (Hi-Load, Amersham Pharmacia) equilibrated with buffer A. A 500 ml linear gradient comprising 0–1000 mM NaCl was used to elute cationic proteins. Fractions containing distinct activities were pooled and concentrated. The concentrated enzyme (7 ml) was loaded onto a 26/70 gel filtration column (Superdex 200 Hi-Load, Amersham Pharmacia) and developed with buffer A containing 300 mM NaCl. Active fractions were pooled and diluted with three volumes of buffer A. Finally, the enzyme was loaded onto a Mono-S column (Amersham Pharmacia) pre-equilibrated with buffer A and eluted with a linear gradient of 100 ml of 0–1000 mM NaCl. The active fractions were concentrated, and stored at 4°C until needed. Total protein concentration in the concentrated Mono-S fraction was adjusted to 1 mg/ml.

2.2. Holliday junction substrates

Oligonucleotides were synthesised and four way DNA junctions assembled as described previously [19], using the following sequences:

Junction Jbm5. This is a branch migrating four way junction with 20 bp arms, assembled from the following oligonucleotides:

strand a, 5′-GCGTTACAATGGAAACTATTCTTGGCAGTTGCATCCAACG
strand b, 5′-CGTTGGATGCAACTGCCAAGAATAGTGTCAGTTCCAGACG
strand c, 5′-CGTCTGGAACTGACACTATTCTTGGCGAATGGTCGTAAGC
strand d, 5′-GCTTACGACCATTCGCCAAGAATAGTTTCCATTGTAACGC

Junction Z28. This fixed four way junction was prepared with arms of 15 bp, assembled from the following oligonucleotides:

strand b, 5′-TCCGTCCTAGCAAGGAGTCTGCTACCGGAA
strand h, 5′-TTCCGGTAGCAGACTAAAAGGTGGTTGAAT
strand r, 5′-ATTCAACCACCTTTTTTTTAACTGCAGCAG
strand x, 5′-CTGCTGCAGTTAAAACCTTGCTAGGACGGA

2.3. Enzyme assays

Assays were carried out using 1 μl (approximately 1 μg total protein) of the partly purified activities, in reaction buffer (20 mM Tris–HCl, pH 7.5, 50 mM NaCl, 15 mM $MgCl_2$) using 10 nM radioactively 5′-^{32}P-labelled junction as a substrate. Calf thymus DNA (0.2 mg/ml) was added as a competitor to minimise non-specific endonuclease activity and DNA binding proteins. Reactions were initiated by the addition of magnesium to the assay mix in 5 μl total volume, and incubated at 60°C for 20 min. Reactions were stopped by the addition of 4 μl formamide loading mix and heating to 95°C, and products were analysed by denaturing gel electrophoresis and phosphorimaging as described previously [20].

3. Results

We showed previously that cell extracts of the crenarchaeote *S. solfataricus* contain two distinct Holliday junction resolving enzyme activities, Hjc and Hje [16]. To extend these studies, we carried out comparative analysis of two highly

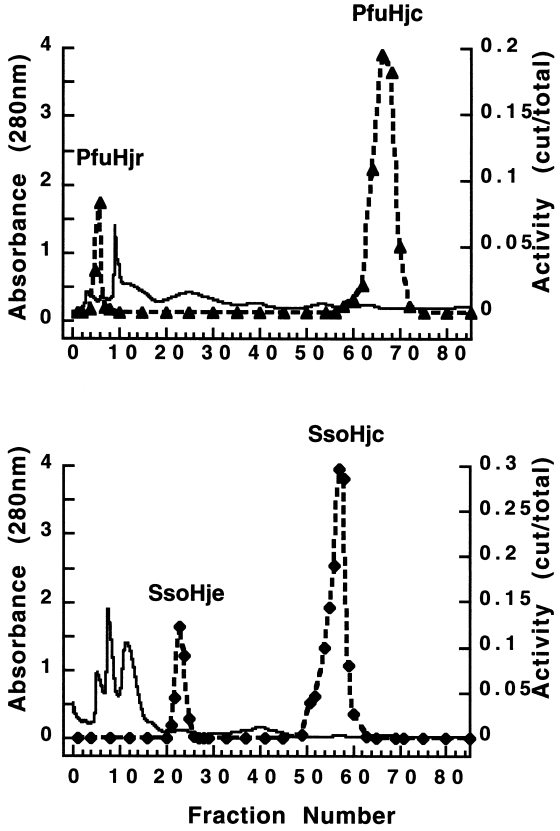

Fig. 1. Detection of Holliday junction resolving enzyme activities in archaeal extracts. 50 g *S. solfataricus* or *P. furiosus* cells were lysed, cleared by centrifugation and fractionated on a high performance SP-Sepharose column as described in Section 2. Fractions were assayed for endonuclease activity using the mobile four way DNA junction Jbm5, and the fraction of junction cleaved by each fraction under standard assay conditions was quantified and plotted (♦ and ▲ for *Sulfolobus* and *Pyrococcus*, respectively). For each species, two peaks of activity were observed. In each case the major activity, corresponding to the Hjc enzyme, eluted late in the gradient. In addition, a secondary activity eluted much earlier in the gradient. The absorbance at 280 nm was monitored, and is represented by the continuous line.

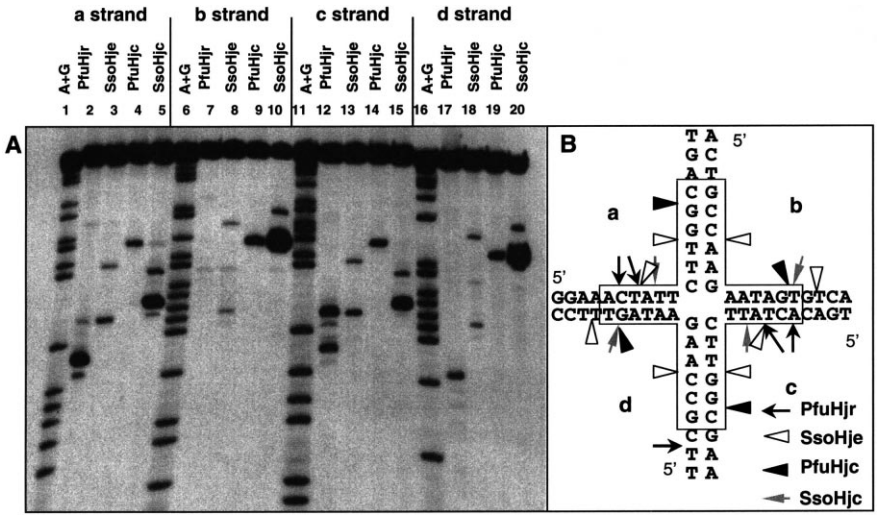

Fig. 2. Cleavage of mobile Holliday junction Jbm5. A: Cleavage of each of the four strands of junction Jbm5 by all four resolving enzyme activities purified from *S. solfataricus* and *P. furiosus* by SP-Sepharose chromatography. Lanes 1–5, Jbm5 strand a; lanes 6–10, Jbm5 strand b; lanes 11–15, Jbm5 strand c; lanes 16–20, Jbm5 strand d. For each strand, the four lanes presented comprise A+G markers, cleavage by *Pyrococcus* Hjr, cleavage by *Sulfolobus* Hje, cleavage by *Pyrococcus* Hjc, and cleavage by *Sulfolobus* Hjc, respectively. B: The sequence of the centre of junction Jbm5 is shown, with the mobile core boxed. Positions cleaved by the four enzymes are indicated by the arrows. Cleavage sites are always paired, allowing junction resolution.

diverged archaeal species, *S. solfataricus* and *P. furiosus*. Cell lysates of *P. furiosus* and *S. solfataricus* P2 were fractionated by ion exchange chromatography, and fractions were assayed for Holliday junction resolving activity using the synthetic mobile four way junction Jbm5 as a substrate. These activities were quantified by estimating the proportion of substrate cleaved in each fraction, yielding a rough estimation of the relative amount of each activity in the cell extracts. In both *Sulfolobus* and *Pyrococcus*, we detected two distinct junction resolution activities, with the Hjc enzyme constituting the bulk of junction resolving activity (Fig. 1). The secondary peak of activity in *Sulfolobus* is due to the Hje enzyme described previously [18], whilst we provisionally assigned the label Hjr (for Holliday junction resolving) to the secondary activity apparent in *P. furiosus*.

We proceeded to characterise the four distinct activities identified in our primary screen in more detail by mapping the sites of cleavage of each arm of the mobile junction substrate Jbm5 (Fig. 2). This junction contains every possible dinucleotide sequence within the mobile homologous core, and previous studies have demonstrated that each resolving enzyme cleaves the junction to give a distinct pattern of products that constitutes a molecular fingerprint for the enzyme [16]. We have shown previously that partly purified and pure recombinant Hjc from *Sulfolobus* have identical properties [16]. All four archaeal enzymes introduced paired cleavages

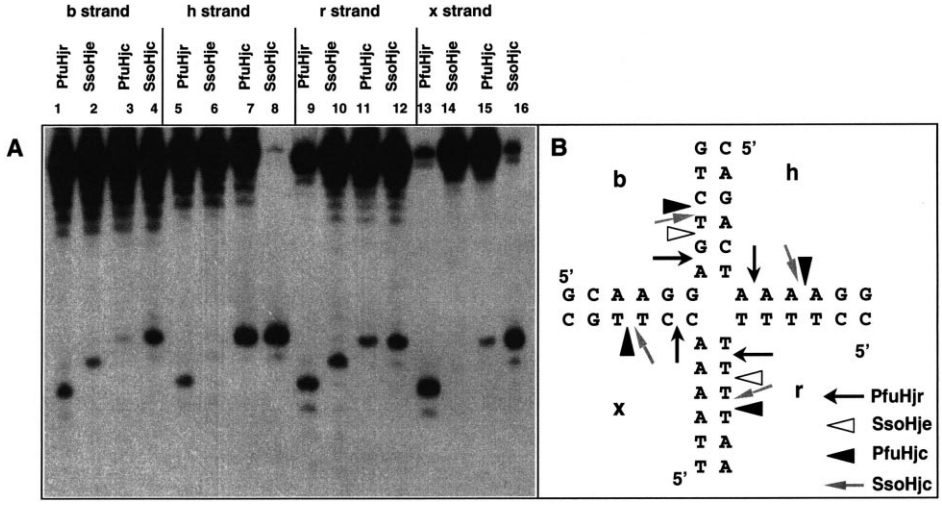

Fig. 3. Cleavage of a fixed four way junction. A: Cleavage of the fixed four way junction Z28 by native Hje and recombinant Hjc are shown analysed by denaturing gel electrophoresis. Lanes 1–4, 5–8, 9–12 and 13–16, cleavage of the b, h, r and x strands of Z28 by *Pyrococcus* Hjr, *Sulfolobus* Hje, *Pyrococcus* Hjc and *Sulfolobus* Hjc, respectively. B: The sequence of the centre of junction Z28 is shown. Positions cleaved by the four enzymes are indicated by arrows. Paired nicks are introduced exclusively in the continuous h and x strands of Z28 by Hje, two nucleotides 3′ of the point of strand exchange. In contrast, both *Pyrococcus* and *Sulfolobus* Hjc cleave all four strands, three nucleotides 3′ of the point of strand exchange. *Pyrococcus* Hjr cleaves all four strands, one nucleotide 3′ of the point of strand exchange.

in opposing arms of the junction, a prerequisite for true resolving enzymes. Clear variations in cleavage patterns were apparent for all four enzymes. Hjc from *Sulfolobus* and *Pyrococcus* introduced coincident nicks in the b and d strands of the junction (Fig. 2, lanes 9+10 and 19+20) but differed in their preferred sites in the a and c strands. The *Sulfolobus* Hje and *Pyrococcus* Hjr activities clearly differ in specificity from the respective Hjc activities, reinforcing that fact that these organisms both contain two distinct Holliday junction resolving enzymes. The failure of Ishino and co-workers [21] to detect the second *Pyrococcus* activity may be due to their choice of assay conditions, as the authors themselves suggest.

In order to analyse the four activities in more detail, we tested their ability to cleave a fixed junction with heterologous arms and a defined point of strand exchange (Fig. 3). The Hjc activities from *Pyrococcus* and *Sulfolobus* each cleaved all four strands of the junction, three nucleotides 3′ of the point of strand exchange, emphasising the point that they are in fact homologous enzymes with broadly similar specificities. *Sulfolobus* Hje gives a very different pattern, cleaving only two strands (b and r), two nucleotides 3′ of the junction centre. Previously, we demonstrated that *Sulfolobus* Hje is specific for strands that adopt a continuous conformation in the stacked form of the junction [18], explaining its tendency to cleave only two of the four possible sites. Lastly, *Pyrococcus* Hjr cleaves all four arms of the junction. Significantly, cleavage occurs one nucleotide 3′ of the point of strand exchange. For fixed junctions, the point of cleavage depends on the orientation of the active site of the enzyme with respect to the junction centre. This suggests Hjr is distinct from both the Hje and Hjc enzymes, with unique characteristics.

4. Discussion

In conclusion, both *Pyrococcus* and *Sulfolobus* contain two distinct Holliday junction resolving enzymes. The two organisms have the cellular Hjc enzyme in common, and in addition have a secondary activity that differs in each case. Archaea thus appear to possess a unique, conserved pathway for homologous recombination of the cellular genome, which includes the RadA protein for strand exchange, and the Hjc protein for junction resolution. Proteins catalysing other steps of the pathway, such as branch migration of the Holliday junction, have yet to be detected.

The additional resolving enzymes in each organism remain unidentified but clearly have different specificities. This is shown most clearly by the pattern of cleavage of the fixed junction Z28 (Fig. 3), which is cleaved with similar specificity by the two Hjc enzymes, but cut at distinct positions with respect to the junction centre by the Hje and Hjr activities, suggesting fundamental differences in the enzymes' orientation of their active sites in the DNA–protein complex. By analogy with the situation in eubacteria and eucarya, the most likely function of these proteins is in viral recombination. Eubacteria possess a cellular resolving enzyme (usually RuvC), and may in addition contain one or more phage resolving enzymes (RusA, T4 endonuclease VII or T7 endonuclease I), whilst pox viruses express a resolving enzyme in the cytosol of host cells in order to catalyse viral recombination and possibly also resolution of the viral genome, which is replicated in tandem arrays linked by cruciform structures [6,22]. A large variety of mobile genetic elements have already been characterised in *Sulfolobus* and other archaea [23]. Our findings suggest that at least some of these elements are capable of homologous recombination, and require a viral Holliday junction resolving enzyme. These predictions may be confirmed by advances in the characterisation of archaeal viruses, and by identification of the secondary Holliday junction resolving activities present in the archaea.

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