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Holliday junction resolvase in *Schizosaccharomyces pombe* has identical endonuclease activity to the CCE1 homologue YDC2

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ABSTRACT

A novel Holliday junction resolving activity has been identified in fractionated cell extracts of the fission yeast *Schizosaccharomyces pombe*. The enzyme catalyses endonucleolytic cleavage of Holliday junction-containing χ DNA and synthetic four-way DNA junctions. The activity cuts with high specificity a synthetic four-way junction containing a 12 bp core of homologous sequences but has no activity on another four-way junction (with a fixed crossover point), a three-way junction, linear duplex DNA or duplex DNA containing six mismatched nucleotides in the centre. The major cleavage sites map as single nicks in the vicinity of the crossover point, 3′ of a thymidine residue. These data indicate that the activity has a strong DNA structure selectivity as well as a limited sequence preference; features similar to the Holliday junction resolving enzymes RuvC of *Escherichia coli* and the mitochondrial CCE1 (cruciform-cutting enzyme 1) of *Saccharomyces cerevisiae*. A putative homologue of CCE1 in *S.pombe* (YDC2_SCHPO) has been identified through a search of the sequence database. The open reading frame of this gene has been cloned and the encoded protein, YDC2, expressed in *E.coli*. The purified recombinant YDC2 exhibits Holliday junction resolvase activity and is, therefore, a functional *S.pombe* homologue of CCE1. The resolvase YDC2 shows the same substrate specificity and produces identical cleavage sites as the activity obtained from *S.pombe* cells. Both YDC2 and the cellular activity cleave Holliday junctions in both orientations to give nicks that can be ligated *in vitro*. The partially purified Holliday junction resolving enzyme in fission yeast is biochemically indistinguishable from recombinant YDC2 and appears to be the same protein.

INTRODUCTION

One fundamental property of DNA metabolism in all organisms is the interaction and exchange of information between homologous chromosomes in genetic recombination and DNA repair. The crucial step in these processes is pairing and exchange of strands between homologous DNA molecules, leading to formation of DNA intermediates joined by a crossover. These structures, known as Holliday junctions, are a central feature of all models for homologous recombination and double-strand break repair (1–3) and have been demonstrated to form both *in vivo* (4,5) and *in vitro* (6,7). Their resolution into separate chromosomes is essential for cell viability. Clearly, cells require enzymes that process Holliday junctions; in particular, specialized endonucleases or resolvases, which cut two strands of the junction close to the crossover site in such a way as to allow segregation and repair of the joined chromosomes.

Two types of junction-resolving endonucleases have been identified and studied extensively in prokaryotes. The bacteriophage enzymes T4 endonuclease VII (8) and T7 endonuclease I (9) recognize and process *in vitro* a variety of branched DNA substrates, including Holliday junctions, and most likely perform diverse functions *in vivo* (reviewed in 10,11). The second type are Holliday junction resolvases required specifically at the late stages of homologous recombination. The RuvC protein of *Escherichia coli* was the first of these enzymes to be identified (12,13) and has been characterized in great detail (reviewed in 11,14,15). In *E.coli* RuvC is part of a pathway for processing Holliday junctions which also involves branch migration promoted by the RuvAB complex (16,17). Both genetic (18–20) and biochemical data (21) provide evidence that branch migration and resolution occur by a concerted mechanism, with RuvABC acting as a complex. Putative homologues of RuvA, RuvB and RuvC have been identified in the genomes of several prokaryotic species and are likely to be ubiquitous in eubacteria. *Escherichia coli* contains at least one other Holliday junction resolvase, RusA, encoded by a defective lambdoid prophage (19,20).

The pathways of processing Holliday junctions in eukaryotes are not well understood, although several resolvase activities have been reported: two activities in the yeast *Saccharomyces cerevisiae*, endo X1 (22,23) and endo X2 (24,25); mammalian activities in calf thymus (26), CHO cells (27), human placenta (28) and HeLa cells (29). Some of these partially fractionated activities have been well characterized (27,30,31) but the enzymes were never purified to homogeneity and their genes remain unknown. The only eukaryotic Holliday junction resolvase identified so far at the molecular level is the yeast cruciform-cutting enzyme CCE1 (32) (shown to be the same as endo X2; 25,33). This enzyme is encoded by the nuclear *CCE1* gene (32) but is localized exclusively in yeast mitochondria (34,35). The biological function of CCE1 appears to be restricted to

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the mitochondrion, as these studies did not detect any effect on nuclear DNA metabolism.

Saccharomyces cerevisiae CCE1 and *E.coli* RuvC share some characteristic biochemical properties (reviewed in 11). Both show high structure selectivity and a sequence preference for their substrates, plus a preference for cutting the 'continuous' strand of synthetic junctions (36,37). The two proteins bind to Holliday junctions as dimers and strongly distort the structure of the junction upon binding (37,38). It has been speculated that CCE1 has a prokaryotic origin (11).

Since no nuclear Holliday junction resolvases have been characterized at the molecular level, the mechanisms and control of the late stages of recombination in eukaryotes remain unknown. Homologous recombination is a very efficient process in the fission yeast *Schizosaccharomyces pombe*, both in mitosis and in meiosis. This organism has highly developed genetics and has been used extensively to study the mechanisms of cell cycle regulation. In addition, molecular features that are common between *S.pombe* and *S.cerevisiae* are likely to be conserved in other eukaryotes, as the two yeast species are highly diverged in evolution. With these considerations in mind, fission yeast cell extracts were fractionated and tested for Holliday junction processing activities using biochemical assays. Here we report the existence and the initial biochemical characterization of a Holliday junction resolving activity in cell extracts of *S.pombe*. Meanwhile, a putative CCE1 homologue of *S.pombe* was identified in the sequence database. We have cloned and expressed in *E.coli* the open reading frame of this protein, YDC2_SCHPO, and shown that recombinant YDC2 is a functional Holliday junction resolvase. The two activities were compared and shown to be indistinguishable. The resolvase present in extracts of *S.pombe* cells is, therefore, most likely the CCE1 homologue.

MATERIALS AND METHODS

Enzymes and materials

Escherichia coli RuvC was a gift from Dr S.West (Imperial Cancer Research Fund) and T4 endonuclease VII was a gift from Prof. B.Kemper (University of Cologne). Enzymes were diluted in 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 100 µg/ml BSA and 10% glycerol. Vent$_R$® DNA polymerase (New England BioLabs), restriction enzymes, T4 polynucleotide kinase, terminal deoxynucleotidyl transferase, T4 DNA ligase, the Klenow fragment of DNA polymerase and proteinase K were obtained from commercial suppliers and used according to their instructions.

Preparation of synthetic DNA substrates and χ structures

Standard molecular biology protocols were followed for all DNA manipulations and for sequencing by the Maxam and Gilbert method (39).

Fully deprotected oligonucleotides were purchased from Genosys and their sequences are given in Table 1. The oligonucleotides were purified by gel electrophoresis on 8% polyacrylamide containing 7 M urea, the DNA bands excised, electroeluted and dialysed against TE buffer. Concentrations were determined by measuring absorbance at 260 nm.

The synthetic substrates were assembled from various combinations of oligonucleotides, as shown in Table 1. For each construct 1 µg of one oligonucleotide was labelled at either the 5′-end with [γ-^{32}P]ATP (3000 Ci/mmol; New England Nuclear) using polynucleotide kinase or the 3′-end with dideoxy[α-^{32}P]ATP (3000 Ci/mmol; Amersham) and terminal transferase. After labelling, 4 µg of each of the other oligonucleotides were added and annealed using a thermal cycler (92°C for 2 min, 65°C for 10 min, 37°C for 10 min and 22°C for 10 min). The assembled substrates were purified by electrophoresis through 8% polyacrylamide as described (40) and dialysed against TE buffer (or water for Maxam–Gilbert sequencing substrates). Concentrations were determined by calculation of the specific radioactivity using the DE81 filter binding method (39).

DNA χ structures were prepared from plasmid intermediates formed *in vivo* by XerC-mediated site-specific recombination (41). *Escherichia coli* strain RM40 and plasmid pSD115 were kindly supplied by Prof. D. Sherratt (University of Oxford). RM40/pSD115 cells were grown at 37°C in LB medium containing 100 µg/ml ampicillin and 50 µg/ml diaminopimelic acid to an A_{600} of 0.5. For induction of XerC expression isopropyl β-D-thiogalactoside (IPTG) was added to a concentration of 1 mM and cells were harvested after 1 h. Plasmid DNA was isolated using Qiagen columns, digested with *Sty*I and *Sca*I and fractionated by electrophoresis on 1% agarose gels. The χ DNA band was excised and electroeluted and labelled at the *Sty*I ends using the Klenow enzyme and [α-^{32}P]dCTP (3000 Ci/mmol; New England Nuclear). The labelled χ DNA was gel purified again as above.

Fractionation of Holliday junction resolvase from *S.pombe* extracts

Wild-type *S.pombe* strain *972h*$^-$ was grown in YE medium (0.5% yeast extract, 3% glucose) at 30°C to late log phase. The cells were harvested, washed with sterile water, re-centrifuged and the cell pellets stored at –70°C.

All fractionation steps were performed in buffer A (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol). All column and dialysis buffers contained the following protease inhibitors (added just before use): *N*-tosyl-L-phenylalanine chloromethyl ketone and *N*-tosyl-L-lysine chloromethyl ketone (both at 50 µg/ml), aprotinin and bestatin (both at 2 µg/ml), leupeptin (0.5 µg/ml), pepstatin (1 µg/ml) and 1 mM phenylmethylsulphonyl fluoride. Fractions from all chromatography steps were concentrated 10- to 50-fold using microcon concentrators (Amicon) and assayed for resolvase activity in the presence of a 500-fold molar excess (in nucleotide residues) of linear double-stranded DNA, as described below. Protein concentrations were determined by the Bradford assay, using BSA as the standard.

For a typical preparation 50–100 g pelleted cells were thawed on ice and resuspended in extraction buffer (25 mM Tris–HCl, pH 7.5, 15 mM $MgCl_2$, 100 mM NaCl, 2 mM NaF, 80 mM β-glycerophosphate, 20 mM EGTA, 1 mM DTT, 0.1% Triton X-100). Immediately before use *p*-nitrophenyl phosphate was added to 15 mM, Na_3VO_4 to 1 mM and the protease inhibitors as listed above. All subsequent manipulations were performed on ice or at 4°C. The cells were broken with glass beads in a pre-chilled bead beater using fifty 10 s bursts. Fresh protease inhibitors were added and the crude supernatant was clarified by two spins (10 000 *g* for 20 min, followed by 200 000 *g* for 60 min). The supernatant (fraction 1) was applied to a DEAE BioGel A column (Macroprep; BioRad) which was developed with a 0.1–1.0 M NaCl gradient. The active fractions eluting between 0.5 and 0.7 M NaCl were pooled to yield fraction 2, dialysed against buffer A and applied to a 5 ml

Table 1. Sequences of oligonucleotides used to make synthetic substrates

Four-way junction X12: oligonucleotides 1, 2, 3 and 4	
1 (61-mer)	GACGCTGCCGAATTCTACCAGTGCC**TTGCTAGGACAT**CTTTGCCCACCTGCAGGTTCACCC
2 (62-mer)	TGGGTGAACCTGCAGGTGGGCAAAG**ATGTCCTAGCAA**TGTAATCGTCAAGCTTTATGCCGTT
3 (63-mer)	CAACGGCATAAAGCTTGACGATTACA**TTGCTAGGACAT**GCTGTCTAGAGGATCCGACTATCGA
4 (62-mer)	ATCGATAGTCGGATCCTCTAGACAGC**ATGTCCTAGCAA**GGCACTGGTAGAATTCGGCAGCGT
Linear duplex L12: oligonucleotides 2 and 5	
5 (62-mer)	AACGGCATAAAGCTTGACGATTACATTGCTAGGACATCTTTGCCCACCTGCAGGTTCACCCA
Four-way junction X0: oligonucleoides 1, 6, 7 and 8	
6 (62-mer)	TGGGTGAACCTGCAGGTGGGCAAAGATGTCCATCTGTTGTAATCGTCAAGCTTTATGCCGTT
7 (63-mer)	CAACGGCATAAAGCTTGACGATTACAACAGATCATGGAGCTGTCTAGAGGATCCGACTATCGA
8 (62-mer)	ATCGATAGTCGGATCCTCTAGACAGCTCCATGTAGCAAGGCACTGGTAGAATTCGGCAGCGT
Three-way junction Y0: oligonucleotides 1, 8 and 9	
9 (62-mer)	TGGGTGAACCTGCAGGTGGGCAAAGATGTCCCATGGAGCTGTCTAGAGGATCCGACTATCGA
Linear duplex L0* (with 6 mismatched bases): oligonucleotides 6 and 10	
10 (62-mer)	AACGGCATAAAGCTTGACGATTACATTGCTAGGACATCTTTGCCCACCTGCAGGTTCACCCA

All sequences are shown 5′→3′. Homologous regions in the core of X12 are shown in bold and the mismatched region of L0* is underlined.

heparin–Sepharose HiTrap column (Pharmacia), using 10 mg protein per chromatography run. The column was developed with a 0.1–1.2 M NaCl gradient. Active fractions (0.8–1.0 M NaCl) were pooled, concentrated 10-fold and dialysed to give fraction 3. This was then applied to a 3 ml phosphocellulose column and developed with a 0.1–1.2 M NaCl gradient. The pooled active fractions (fraction 4) contained a nuclease activity which was removed by passage through a 1 ml Octyl Sepharose hydrophobic HiTrap column (Pharmacia) in buffer A containing 1 M NaCl and the flow-through (fraction 5) was further fractionated by Mono S FPLC. The cutting activity eluting at ~0.9 M NaCl was pooled to yield fraction 6.

Fractions were stored at –20°C in 50% glycerol and retained their cutting activity for up to 4 weeks. Flash freezing and storage of fractions at –70°C extended their life, but repeated freezing and thawing was detrimental.

Cloning and expression of *S.pombe* YDC2

The YDC2_SCHPO open reading frame was amplified from *S.pombe 972h*⁻ chromosomal DNA by PCR. Primer sequences were: 5′-oligonucleotide, 5′-ATATATCATATGGCTACTGTGAA-ACTTAGTTTTTTACAGC; 3′-oligonucleotide, 5′-ATATCTCGA-GCTATTGTTTCAGAAACTGTTTGCAAAAGTTTC. An *Nde*I restriction site was engineered at the start codon of the sequence in-frame with the start codon in the T7 expression vector pET21a(+) (Novagen) and a *Xho*I restriction site was introduced at the 3′-end of the sequence, after the natural stop codon. The PCR product was cleaved with *Nde*I and *Xho*I, cloned into pET21a(+) and the recombinant plasmid pET21-YDC2 used to transform *E.coli* strain BL21(DE3).

For expression of the YDC2 protein the cells were grown in LB medium containing 100 µg/ml ampicillin at 37°C to an A_{600} of 0.5 and induced with IPTG (1 mM final concentration) for 4 h at 30°C. The induced cells were harvested, re-suspended in TED buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.5 mM DTT, 10% glycerol) and lysed by treatment with lysozyme and 0.1% Triton X-100 in 0.1 M NaCl as described (42). The lysate, containing only a fraction of the overexpressed protein, was subjected to column chromatography on phosphocellulose, heparin–Sepharose HiTrap and Mono Q FPLC.

Assays of resolvase activity

The cutting of synthetic substrates was assayed by adding up to 2 µl of the fractions to 10 µl reaction mixtures containing 1–5 ng X12

DNA (typically ~10^4 c.p.m.) in 50 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM KCl, 1 mM DTT and 100 µg/ml BSA. Single-stranded virion ϕX174 circular DNA (New England BioLabs) or ϕX174 form I DNA cut with *Hin*fI (Promega) were used as competitors when required. Control reactions (at 37°C) contained 5 µg/ml RuvC or 100 U T4 endonuclease VII. Reactions were incubated at 30°C for 60 min and stopped by addition of EDTA. Samples were extracted with phenol/chloroform and precipitated with ethanol before electrophoresis on 8% neutral or 12% denaturing polyacrylamide gels. The gels were dried and autoradiographed on X-OMAT XAR-5 or BioMax MS-1 films from Kodak.

For the ligation experiments a cutting assay was performed as above and one half of the reaction mixture was stopped and processed as described. To the remaining sample 10 U T4 DNA ligase and ATP (1 mM final concentration) were added; the reactions were further incubated for 30 min at 37°C, then stopped and processed as before.

Assays for cutting of χ structures were as described for the synthetic substrates using ~10^4 c.p.m. ^{32}P-labelled DNA per assay. Reactions (20 µl) were stopped and deproteinized by adding 5 µl 5× stop buffer (0.5% proteinase K, 100 mM Tris–HCl, pH 7.5, 200 mM EDTA, 2.5% SDS), followed by incubation for 15 min at 37°C. Products were analysed by 1.2% agarose gel electrophoresis and autoradiography.

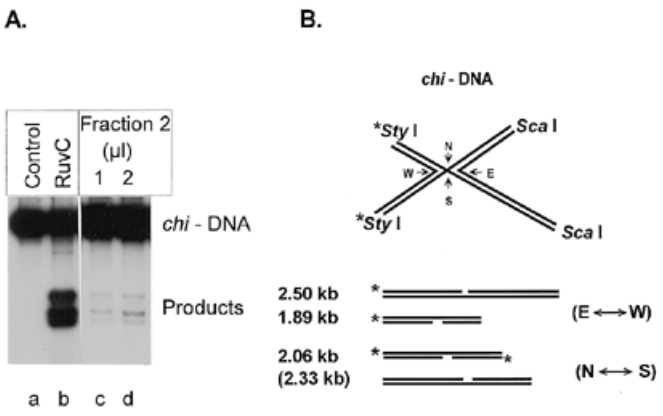

Figure 1. Activity in fractionated *S.pombe* extract cleaves Holliday junction-containing χ DNA. (**A**) Reactions contained ^{32}P-labelled χ DNA in 20 µl reaction buffer with 1 or 2 µl fraction 2 or 50 ng RuvC, as indicated. After incubation for 20 min at 30°C (or 37°C for RuvC) reactions were stopped as described and analysed by 1.2% agarose gel electrophoresis followed by autoradiography. (**B**) Schematic presentation of χ DNA structures, labelled at the *Sty*I ends. Resolution of the Holliday junction in two orientations (north–south and east–west) produces four nicked linear DNA species (three of them labelled) of the sizes indicated.

DNA binding assays

Reactions contained ~5 ng labelled synthetic DNA and increasing amounts of YDC2 in 10 µl binding buffer (50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 1 mM DTT, 100 µg/ml BSA, 5% glycerol) and a 500-fold molar excess of competitor DNA. The ratio reflects DNA concentrations in nucleotide residues. The reactions were incubated on ice for 20 min, gel loading buffer was added and the samples loaded directly onto 6% neutral polyacrylamide gels in TBE (43). Electrophoresis was at 10 V/cm at 4°C with continuous buffer re-circulation. The gels were dried and autoradiographed.

RESULTS

Fractionated cell extracts of the fission yeast *S.pombe* contain a Holliday junction resolvase

Whole cell extracts of *S.pombe* were fractionated by DEAE BioGel chromatography and assayed using the synthetic four-way junction X12 (Table 1). Fractions eluting at ~0.6 M NaCl (fraction 2) contained an activity that cut the synthetic junction. As the use of junction X12 could be misleading in the presence of other nucleases and has been questioned (44), the active fractions were also assayed using Holliday junction-containing χ structures, obtained from XerC-mediated site-specific recombination (41,45). As shown in Figure 1, fraction 2 cut the χ structures to give all the expected labelled products in ratios consistent with resolution of the junction in both orientations. The activity detected was, therefore, a true and novel Holliday junction resolvase.

The substrate preference of the yeast activity was examined using additional synthetic substrates as shown in Figure 2. The activity in fraction 2 clearly cut the four-way junction X12, which contains a 12 bp core of homologous sequences and can undergo limited branch migration (Fig. 2, lane c), but the enzyme did not cleave junction X0, which has a fixed crossover point (Fig. 2, lane p). As junctions X0 and X12 share identical sequences in two of their arms, it is possible that discrimination between the two junctions was determined by the location of these sequences relative to the crossover point. Fraction 2 had no activity on the 62 bp duplex DNA L12, which contains the same sequences as two arms of junction X12 (Fig. 2, lane g), or L0*, the duplex with six mismatched nucleotides in the middle (Fig. 2, lane k).

The specificity of the resolvase activity was further confirmed by testing duplex and single-stranded DNAs as competitors. The activity was not affected by the presence of a 1000-fold molar excess of linear duplex competitor DNA (Fig. 2, lane e). However, fraction 2 was inhibited by the same molar excess of ϕX174 circular single-stranded DNA (Fig. 2, lane d). Further competition experiments using this DNA or oligonucleotide 2 (Table 1) showed that the inhibitory effect of the phage DNA was ~10-fold higher than that of the 62mer oligonucleotide and both the yeast activity and RuvC were inhibited to the same extent (data not shown). Various forms of secondary structures in the circular single-stranded DNA are likely to mimic the branch points of a Holliday junction and thus compete with the synthetic four-way junctions, as seen here.

The high specificity of the *S.pombe* activity for junction DNA was exploited throughout the fractionation scheme, where fractions were assayed both with and without competitor duplex DNA, which helped to identify the peak of Holliday junction resolvase in the presence of other nuclease activities. Fractions more highly enriched in the resolvase were used in the experiments shown below. However, even the purest active fractions still contained numerous protein bands when analysed by SDS–PAGE and silver staining (data not shown). The stability of the enzyme progressively deteriorated in the course of purification, possibly due to proteolysis and/or dilution effects. Fractionation of 300 g wet cells by five chromatography steps yielded fraction 6, which contained ~50 µg total protein. Scaling up of the preparation combined with faster chromatography steps would be required to achieve further purification.

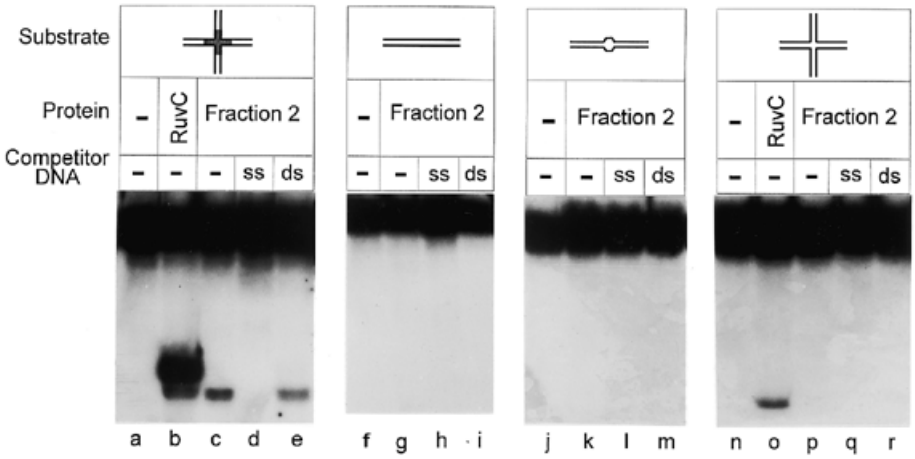

Figure 2. Cleavage of synthetic DNA substrates by fractionated yeast extract. Reactions containing 5 ng DNA (3′-labelled in one strand with ^{32}P) in 20 µl reaction buffer were incubated for 60 min with 2 µl fraction 2 (at 30°C) or 50 ng RuvC (at 37°C), as indicated. A 1000-fold molar excess of either circular single-stranded DNA (ss) or linear duplex DNA (ds) was present where shown. The substrates used were four-way junction X12 (lanes a–e), duplex L12 (lanes f–i), duplex L0* containing a central 6 bp mismatch (lanes j–m) or four-way junction X0 (lanes n–r), drawn schematically for each set of experiments. The reactions were stopped by addition of formamide gel loading buffer and analysed by denaturing 12% polyacrylamide gel electrophoresis followed by autoradiography.

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CCE1    MSTAQKAKILQLIDS CCQNAKSTQLKSLSF VIGAVNGTTKEAKRT YIQEQCEFLEKLRQQ KIREGRINILSMDAG VSNFAFSKMQLLNND   90
                        :   :.    :  . : ..  ..        :.: ..     :      .: .: : . :: .      : :
YDC2    --------------- -----MATVKLSFLQ HICKLTGLSRSGRK- -----DELLRRIVDS PIYP-TSRVLGIDLG IKNFSYCFASQ-NED   62

CCE1    PLPKVLDWQKINLEE K-FFQNLKKLSLNPA ETSELVFNLTEYLFE SMPIPDMFTIERQRT RTMSSRHILDPILKV NILEQILFSNLENKM  179
            .  :   :: : :  .       ..:     .: ..:   : :  .  : .. .:::: :.     : .  :.: :.:: .:.
YDC2    SKVIIHNWSVENLTE KNGLDIQWTEDFQPS SMADLSIQLFNTLHE KF-NPHVILMERQRY RSG-IATIPEWTLRV NMLESMLYALHYAEK  150

CCE1    KYTNKIPNTSKLRYM VCSSDPHRMTSYWCI PREETPTSSKKLKSN KHSKDSRIKLVKKIL STS-ILEGNSTSSTK LVEFIGVWNNRIRNA  268
        .  : : .     .. .  : :    :::         . ::: :       ::. .:: ..     ::  :         : .  :::  :
YDC2    R--NSIEQKIQYPFL LSLS-PKSTYSYWAS VLNTKASFSKK-K-- -----SRVQMVKELI DGQKILFEN------ -EEALYKWNNGSR--  220

CCE1    LTKKKSFKLCDILEI QDNSGVRKDDDLADS FLHCLSWMEWLKNYE SITELLNSKTLVKTQ FGQVFEFCENKVQKL KFLQNTYNND       353
              ::              : ::.:::  :    :: :                    : .     ::    : : :
YDC2    ----VEFK------- -------K-DDMADS ALIASGWMRWQA--- --------------Q LKHYRNFCK---QFL KQ--------       258
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Figure 3. Sequence comparison of *S.cerevisiae* CCE1 and *S.pombe* YDC2. The figure shows an alignment of the CCE1 protein sequence (353 amino acids) with the open reading frame of YDC2 (258 amino acids) based on the ClustalW 1.7 multiple sequence alignment program (54). Identical amino acids are indicated with a colon and conserved residues with a dot.

The cleavage sites of the yeast resolvase were mapped at high resolution on all four strands of junction X12, as shown in the next section (see Fig. 6). The activity produced symmetrical nicks in strands of the same polarity, close to the crossover point, and when reactions were analysed by native PAGE DNA products with the mobility of duplex DNA (L12) were observed (not shown).

All these results demonstrate that the activity detected in *S.pombe* is a *bona fide* Holliday junction resolvase and, like RuvC and CCE1, shows high structure selectivity and limited sequence preference.

YDC2, a putative *S.pombe* homologue of *S.cerevisiae* CCE1, is a functional Holliday junction resolvase identical to the activity in yeast extracts

While purification of the resolvase activity was in progress a search in the *S.pombe* sequence database revealed an open reading frame on chromosome I which encoded a predicted 30.2 kDa protein (YDC2_SCHPO) showing ~30% identity to *S.cerevisiae* CCE1. The alignment of CCE1 and its putative *S.pombe* homologue (Fig. 3) shows a number of short conserved regions.

As this putative CCE1 homologue was a strong candidate for the activity identified in the yeast cell extracts, the open reading frame was amplified from genomic DNA using PCR and cloned into the *E.coli* expression vector pET21a(+). A recombinant protein of ~29.5 kDa was greatly overexpressed in this system, as observed by SDS–PAGE (not shown). A fraction of this protein was present in the soluble lysate obtained from induced BL21(DE3)/pET21-YDC2 cells, which also exhibited a prominent Holliday junction resolving activity. Recombinant YDC2 was subsequently purified to near homogeneity, as shown (Fig. 4A, lane b).

Binding of YDC2 to junction DNA was examined by gel retardation experiments. Figure 4B shows the binding of YDC2 to junction X12 in the presence of a 500-fold molar excess of linear duplex DNA. Accumulation of a protein–junction complex was observed in reactions containing increasing amounts of protein. A second heavier protein–DNA complex appeared at higher protein

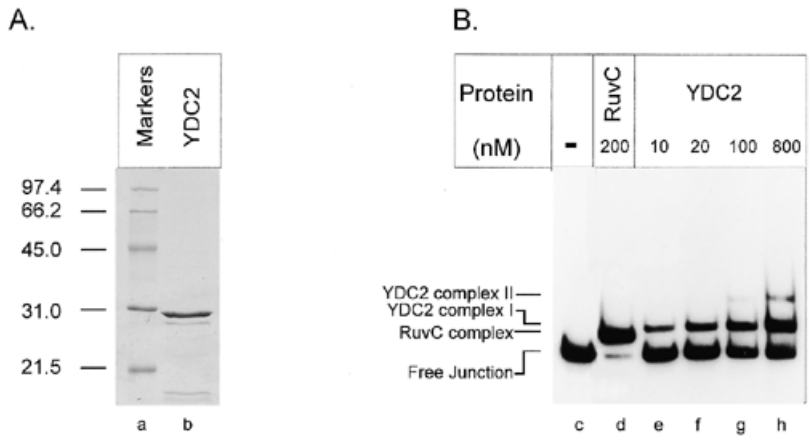

Figure 4. YDC2 binds specifically to a synthetic four-way junction. (**A**) SDS–PAGE of YDC2 (2.4 µg) on a 12% gel, stained with Coommassie brilliant blue. M_r standards were 'low range' from BioRad. (**B**) The indicated amounts of YDC2 were incubated for 20 min on ice with ^{32}P-labelled junction X12 (3 nM), pre-mixed with a 500-fold excess of linear duplex DNA, as described. A control reaction contained 200 nM RuvC. The samples were separated on a 6% polyacrylamide gel and visualized by autoradiography.

concentrations (Fig. 4B, lane h). The main YDC2–junction complex migrated slightly behind the RuvC–junction complex used as a control. The 19 kDa RuvC binds to Holliday junctions as a dimer (38), as does CCE1 (37). It is most likely that YDC2 also binds the junction as a dimer.

The biochemical properties of recombinant YDC2 and the yeast cellular activity were compared in the following experiments. The substrate specificities of the two enzymes were tested using synthetic DNA substrates as shown in Figure 5. Both enzymes readily cut junction X12 producing identical products (Fig. 5, lanes c and d). No cutting was observed using junction X0, the three-way junction Y0 (Fig. 5, lanes g, h, k and l) or both linear duplexes L12 and L0* (not shown).

The cleavage sites of the cellular enzyme and YDC2 were mapped on all four strands of junction X12 (Fig. 6). Both YDC2 and the cellular activity cut each strand at identical sites, producing the same cleavage patterns of major and (on some strands) minor sites. Strands 2 and 4 were cleaved more efficiently than 1 and 3 in this experiment. As shown schematically in Figure 6B, cuts were at symmetrical points on strands of the same polarity, which is one of the characteristics of a Holliday junction resolvase (46). A second property, that of producing ligatable ends, was demonstrated by the experiments shown in Figure 7. Nicks produced by both recombinant YDC2 and fraction 4 were efficiently repaired *in vitro* by T4 DNA ligase (Fig. 7B and C respectively). Nicks on all four strands were ligated (Fig. 7C), consistent with these being resolution sites.

Taken together, these results demonstrate that recombinant YDC2 is a functional homologue of CCE1 and that the partially purified yeast enzyme has identical biochemical properties as YDC2.

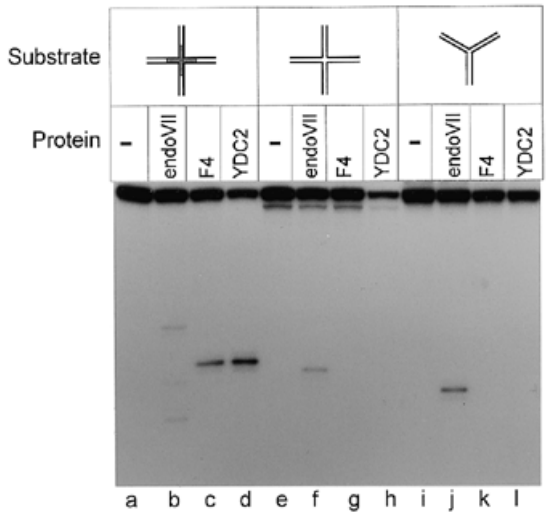

Figure 5. The endonuclease activities of YDC2 and *S.pombe* fraction 4 are highly specific for the four-way junction X12. Reactions with 5 ng DNA were incubated in 10 µl reaction buffer either alone (lanes a, e and i) or with 100 U T4 endonuclease VII (endoVII, lanes b, f and j), 2 µl fraction 4 (F4, lanes c, g and k) or 0.3 µg YDC2 (lanes d, h and l). The substrates were junction X12, junction X0 or the three-way junction Y0, as drawn schematically. Reactions with the *S.pombe* activities also contained a 500-fold molar excess of linear duplex DNA. After incubation for 60 min at 30°C (or 37°C for endo VII) the reactions were stopped as described and analysed by denaturing 12% polyacrylamide gel electrophoresis followed by autoradiography.

DISCUSSION

A novel eukaryotic Holliday junction resolvase was identified in *S.pombe* cell extracts, using a biochemical approach. The cellular activity was shown to be a true resolvase, as it cleaved Holliday junction-containing χ DNA in both orientations. In addition, the activity cut one synthetic four-way junction (X12) but had no activity on another (X0), duplex DNA, mismatched regions or a three-way junction. In parallel, the putative CCE1 homologue of *S.pombe* was cloned and expressed in *E.coli* and shown to be a functional Holliday junction resolvase *in vitro*. The two enzymes were biochemically indistinguishable in all our experiments: they showed the same substrate specificity, nicked identical sites in synthetic junctions and produced ends which were efficiently repaired by DNA ligase. The alignment of the cleavage sites was a particularly strong indication that the two activities were identical. Despite their limited sequence selectivity, different

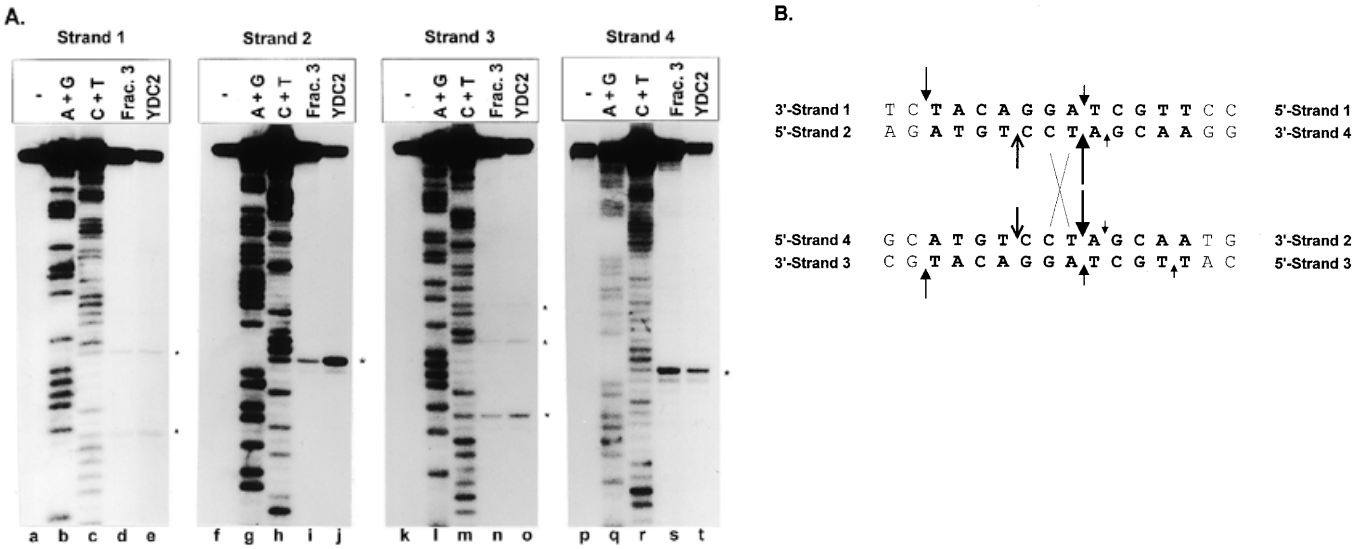

Figure 6. YDC2 and *S.pombe* fraction 3 cut each strand of junction X12 at identical sites. (**A**) Four preparations of junction X12, each labelled uniquely at the 3′-end of one strand, as indicated, were used in separate reactions containing 5 ng DNA and ~0.5 µg YDC2 or 2 µl fraction 3 in 20 µl reaction buffer. Control reactions contained no added protein. Samples were incubated at 30°C for 60 min and processed as described. The products were resolved by denaturing 12% polyacrylamide gel electrophoresis and visualized by autoradiography. The bands produced are marked with asterisks. Maxam–Gilbert sequencing reactions specific for purines (A + G) or pyrimidines (T + C) are shown for each labelled junction. (**B**) Schematic presentation of the central sequences of junction X12 showing the cleavage sites identified above. The cleavage sites of the yeast enzymes are represented by filled arrows of varying sizes to illustrate the differences in cutting efficiencies. Open arrows show the main RuvC cleavage sites for comparison (46). The region of homology is shown in bold, with the crossover positioned arbitrarily.

resolvases produce a very specific pattern of major and minor cleavage sites on synthetic junctions (Figs 2 and 5; 37,47). Most probably this is a reflection of subtle differences in the interplay between the structure and sequence requirements of these enzymes. The highly individual spectrum of cleavage sites could, therefore, be used as evidence for identity.

While this manuscript was in preparation two other laboratories reported similar results, characterizing the putative protein YDC2 of *S.pombe* as a functional Holliday junction resolvase (48,49). In both studies YDC2 was expressed and purified from *E.coli* as a fusion protein. Although clearly active, the fusion protein did not complement a *ruvC rusA* mutant strain of *E.coli* as efficiently as full-length YDC2 alone (49). Whitby and Dixon also characterized an activity from fractionated *S.pombe* extracts which was biochemically identical to YDC2 (49). Although a different fractionation scheme was used the activity they obtained from yeast cells is very similar to the enzyme described here.

Like CCE1, the *S.pombe* enzyme(s) has a strong structure specificity and some sequence preference. The mapping of the cleavage sites showed that the main cuts were produced 3′ of a thymidine residue, consistent with the cleavage sites mapped in the other two laboratories (48,49). A preliminary consensus sequence 5′-CXT↓-3′ could be derived from our data. Clearly, this sequence needs to be present in a specific structural context, such as distance from the crossover point and overall conformation of the junction. Comparison with the sequence preferences of RuvC (5′-$^{A}/_{T}$TT-$\downarrow^{G}/_{C}$-3′) (50) and CCE1 (5′-CT↓-3′) (37) shows that these enzymes also cut 3′ of a thymidine residue. This implies that some feature of the active site is common to resolvases from diverse sources.

Conservation of CCE1 between the two yeast species is evidence for a conserved pathway playing an important biological role. In *S.cerevisiae* CCE1 is localized in mitochondria and appears to be involved only in metabolism of the mitochondrial genome. This is likely to be the case in *S.pombe* and we are undertaking further experiments in this regard. CCE1 homologues are likely to be present in other eukaryotes, especially plants which harbour both mitochondrial and chloroplast genomes. It is of particular interest to search for a human CCE1 homologue and to study its effect on mitochondrial genome stability, as mutations and deletions in mtDNA are the molecular basis of some human diseases and may be one of the mechanisms of ageing (reviewed in 51).

The role for a Holliday junction resolvase in maintenance of the mitochondrial genome is not fully understood. Clearly, CCE1 of *S.cerevisiae* resolves mtDNA recombination junctions *in vivo*, as shown directly by accumulation of mtDNA junctions in *cce1* (*mgt1*) mutants (52). It has been proposed that several mitochondrial genomes linked via recombination junctions constitute the mtDNA heritable unit in *S.cerevisiae*, whose size is affected directly by CCE1 (52). This may be a specialized adaptation of a more general role played by junction resolvases during DNA replication, as Holliday junctions were recently shown to accumulate in replication mutants of *S.cerevisiae* (53). A YDC2 deletion strain of *S.pombe* should throw more light on the biological role of this protein.

A YDC2-lacking strain will also open the way to look for nuclear Holliday junction processing activities in *S.pombe*, which are likely to be masked by the mitochondrial activity. The search for enzymes involved in the late stages of recombination in eukaryotes is particularly important, as no identifiable RuvA, RuvB or RuvC homologues can be found in the eukaryotic DNA sequences available at present. It may turn out that Holliday junctions are processed in eukaryotes via novel mechanisms, distinct from the mechanisms and proteins identified in prokaryotes.

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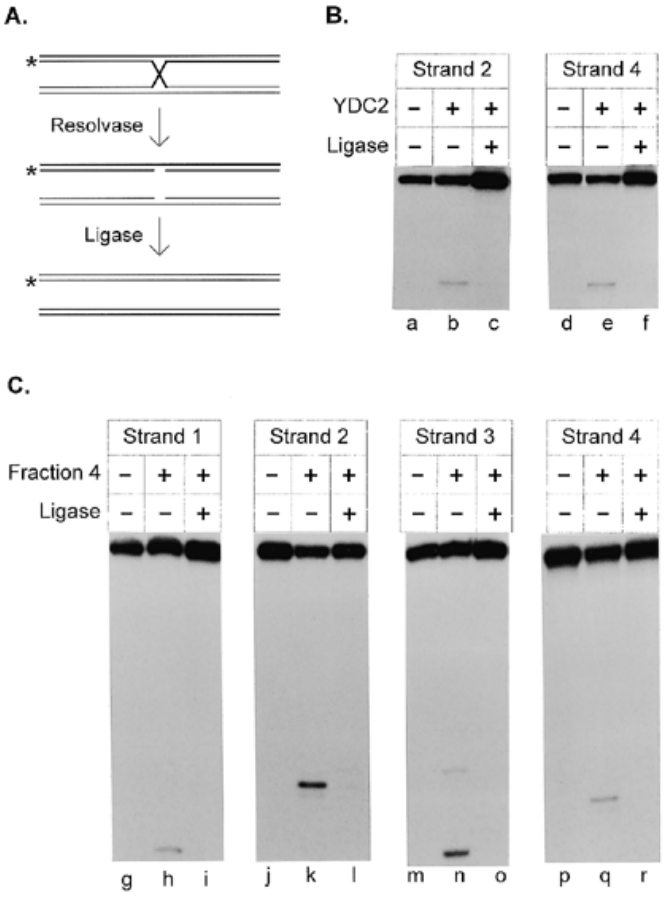

Figure 7. Repair by DNA ligase of DNA nicks produced by YDC2 and *S.pombe* fraction 4. (**A**) Schematic diagram of a four-way junction, labelled in one strand (*). Resolution of the junction via a cut on the labelled strand yields a much shorter labelled product. The full-length strand is restored when the nick is repaired by DNA ligase. (**B**) Ligation of nicks produced by YDC2 on strands 2 and 4 of junction X12. (**C**) Ligation of all four strands of X12 cut by fraction 4. Cutting assays contained 5 ng junction X12 labelled uniquely at the 3′-end of the indicated strand and 2 µl either YDC2 or fraction 4 as described previously. After 60 min at 30°C one half of each reaction was stopped while the remainder was treated with 10 U T4 DNA ligase in the presence of 1 mM ATP for a further 30 min at 37°C. The reactions were analysed by denaturing 12% polyacrylamide gel electrophoresis followed by autoradiography.

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