Two distinct DNA ligase activities in mitotic extracts of the yeast *Saccharomyces cerevisiae*  

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**ABSTRACT**  
Four biochemically distinct DNA ligases have been identified in mammalian cells. One of these enzymes, DNA ligase I, is functionally homologous to the DNA ligase encoded by the *Saccharomyces cerevisiae* *CDC9* gene. Cdc9 DNA ligase has been assumed to be the only species of DNA ligase in this organism. In the present study we have identified a second DNA ligase activity in mitotic extracts of *S.cerevisiae* with chromatographic properties different from Cdc9 DNA ligase, which is the major DNA joining activity. This minor DNA joining activity, which contributes 5–10% of the total cellular DNA joining activity, forms a 90 kDa enzyme–adenylate intermediate which, unlike the Cdc9 enzyme–adenylate intermediate, reacts with an oligo (pdT)/poly (rA) substrate. The levels of the minor DNA joining activity are not altered by mutation or by overexpression of the *CDC9* gene. Furthermore, the 90 kDa polypeptide is not recognized by a Cdc9 antiserum. Since this minor species does not appear to be a modified form of Cdc9 DNA ligase, it has been designated as *S.cerevisiae* DNA ligase II. Based on the similarities in polynucleotide substrate specificity, this enzyme may be the functional homolog of mammalian DNA ligase III or IV.  

**INTRODUCTION**  
DNA ligation is an essential step in DNA replication, DNA excision repair and genetic recombination. Studies in prokaryotes such as *Escherichia coli* suggest that the cellular requirement for DNA ligation is fulfilled by a single DNA ligase (1). In contrast, two and four distinct species of DNA ligase have been characterized in extracts from *Drosophila* (2) and mammalian cells (3,4), respectively. DNA ligase II appears to be derived from DNA ligase III by a proteolytic mechanism (5–7). Thus, the three human genes encoding DNA ligases identified so far can account for the four characterized enzyme activities (3–5,8).  

Mammalian DNA ligase I is the most extensively studied eukaryotic DNA ligase. This enzyme appears to be required for the joining of Okazaki fragments at the replication fork (9–12) and is also involved in some pathways of DNA repair (12,13). Human DNA ligase I cDNA complements the conditional lethal phenotype of a *S.cerevisiae cdc9* mutant defective in DNA ligase (14). Not surprisingly, mammalian DNA ligase I and yeast Cdc9 DNA ligase possess identical polynucleotide substrate specificities and so Cdc9 can be considered to be DNA ligase I of *S.cerevisiae* (4,15,16). Enzymes with biochemical properties similar to mammalian and yeast DNA ligase I have also been purified from extracts of *Drosophila* (2,17,18), *Xenopus laevis* (19) and trypanosomes (20).  

Mammalian DNA ligase II was initially described as a minor activity in extracts of calf thymus that bound more firmly to hydroxyapatite than the major activity, DNA ligase I (21). The 70 kDa DNA ligase II has been purified to homogeneity from both bovine thymus and liver (7,22). It is immunologically distinct from DNA ligase I (22,23) and also possesses different catalytic properties, including the ability to join oligo (pdT) molecules hybridized to a poly (rA) template (4,24). The human *LIG3* and *LIG4* genes encode DNA ligases with calculated molecular weights of ~100 kDa. These enzymes are also active with the oligo (pdT)/poly (rA) substrate (3–5,8). Amino acid sequencing of peptides from bovine DNA ligases II and III revealed the presence of identical amino acid sequences within these polypeptides (6,7). Subsequent cloning experiments resulted in the identification of a cDNA whose translated open reading frame contains regions homologous to all the DNA ligase II and III peptides (5–8). The absence of different sized transcripts in tissues such as the liver, in which DNA ligase II is the predominant activity, suggests that DNA ligases II and III are not generated by an alternative splicing mechanism (5,7). While the formal possibility that DNA ligase II is encoded by a distinct gene cannot be ruled out, it appears more likely that DNA ligase II is derived from DNA ligase III by proteolytic processing.  

The interaction between 103 kDa DNA ligase III and the human DNA strand-break repair protein XRCC1 implicates DNA ligase III in the repair of DNA single-strand breaks which arise as a consequence of DNA damage by alkylating agents and ionizing radiation (25–27). A 96 kDa form of DNA ligase III with a C-terminus distinct from that in the 103 kDa form of the enzyme is produced by an alternative splicing event which is restricted to...  

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male germ cells undergoing mitotic recombination (28). The 96 kDa protein does not interact with XRCC1 and thus may play a specific role in the completion of mitotic recombination with protein partners other than XRCC1 (28). The open reading frame encoding DNA ligase IV was identified by screening a human EST database with a peptide sequence that is conserved in eukaryotic DNA ligases (8). At the present time, the biological function(s) of DNA ligase IV is unknown. In summary, three mammalian LIG genes encode distinct polypeptides designated DNA ligases I, III and IV. Two species of DNA ligase III exist as a consequence of alternative processing of the LIG3 transcript whilst DNA ligase II appears to be derived from DNA ligase III by proteolysis.

A second DNA ligase activity that is clearly distinct from the replicative DNA ligase has been partially purified from Drosophila pupae and embryos (2,29), indicating that multiple species of DNA ligase occur in other multicellular eukaryotes. At present there is no compelling biochemical or genetic evidence for DNA ligases in other lower eukaryotes besides the previously described Cdc9 DNA ligase of S.cerevisiae and the Cdc17 DNA ligase of S.pombe (30,31). To address this question we have begun to investigate the repertoire of DNA ligases in S.cerevisiae. In this report we describe the partial purification of a DNA ligase activity from extracts of haploid S.cerevisiae cells. Since this activity is not recognized by a Cdc9 antibody and is not affected by a cdc9 mutation, we conclude that it is distinct from Cdc9 DNA ligase (DNA ligase I). Consequently, we have designated this activity, which may be functionally homologous to mammalian DNA ligase III or IV, as S.cerevisiae DNA ligase II.

MATERIALS AND METHODS

Plasmids and strains

The S.cerevisiae strain, BJ2168 (MATa prc1-407 prb1-1122 pep4-3 leu2 trpl ura3-52) was from the Yeast Genetic Stock Center, Berkeley, CA. The S.cerevisiae strains, M1-2B (CDC9 ura3 trpl1) and L94-4D (cdc9-7 ura3 trpl1) and the plasmid p207Sclig1BH (32), a derivative of pJDB207, were supplied by Dr L. H. Johnston, Laboratory of Cell Propagation, National Institute of Medical Research, UK. Strain BJ2168 and BJ2168 harboring the plasmid p207Sclig1BH were grown in a 60 l New Institute of Medical Research, UK. Strain BJ2168 (MATa cdc9-7 ura3 trpl1) and the plasmid pKK223-3-RLGX was supplied by Dr E. M. Phizicky, Department of Biochemistry, University of Rochester. Expression of the LIG2 genes encoding DNA ligase IV was from extracts of haploid S.cerevisiae cells. Since this activity is not recognized by a Cdc9 antibody and is not affected by a cdc9 mutation, we conclude that it is distinct from Cdc9 DNA ligase (DNA ligase I). Consequently, we have designated this activity, which may be functionally homologous to mammalian DNA ligase III or IV, as S.cerevisiae DNA ligase II.

Preparation of polynucleotide substrates

Oligonucleotides (pdT)16, (dT)16 and (trA)12-18 and polynucleotides (dA), (trA) and (dT) were purchased from Pharmacia. Dephosphorylated oligonucleotides were labeled and annealed with the appropriate polynucleotide (16). A DNA substrate that contains a single defined nick was prepared by annealing two oligonucleotides, a labeled 16mer and an 18mer with a complementary 34mer as described previously (16).

DNA ligase assays

Reaction mixtures (60 µl) contained 50 mM Tris–HCl (pH 8.0), 10 mM MgCl2, 5 mM dithiothreitol (DTT), 1 mM ATP, 50 µg/ml bovine serum albumin, polynucleotide substrate (20 000 c.p.m.) and a limiting amount of DNA ligase (4,16). Incubations were at 20°C for 15 min. The conversion of 32P-labeled phosphomonoesters to alkaline phosphomonoesters was measured using bacterial alkaline phosphatase (Sigma). One unit of DNA ligase activity catalyses the conversion of 1 pmol terminal phosphate residues to a phosphatase-resistant form in 15 min at 20°C.

Formation of DNA ligase–adenylate complex

Reaction mixtures (20 µl) contained 60 mM Tris–HCl (pH 8.0), 10 mM MgCl2, 5 mM DTT, 50 µg/ml bovine serum albumin, 0.5 µCi [γ-32P]ATP (300 Ci/mmol, Amersham) and DNA ligase. Incubations were at room temperature for 15 min. After the addition of 5 µl of sodium dodecyl sulfate (SDS) sample buffer, reaction mixtures were heated at 90°C for 10 min. Proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) (34). Gels were fixed for 10 min in 10% acetic acid and dried. Adenylylated proteins were detected by autoradiography.

To examine the reactivity of the enzyme–adenylate intermediate, the adenylylation reaction was performed as described above. Aliquots (10 µl) were incubated with 0.8 µg unlabelled polynucleotide or with 20 nmol sodium pyrophosphate for 2 h at 20°C. Reactions were stopped by the addition of SDS sample buffer and adenylylated polypeptides were detected as above.

Purification of yeast DNA ligase II

Protein purification was carried out on ice or at 4°C. All buffers contained a cocktail of protease inhibitors (35). Protein concentrations were measured by the method of Bradford using bovine serum albumin as the standard (36). A cleared lysate (510 ml, 6.12 g) was prepared from 200 g wet weight of BJ2168 cells and fractionated by phosphocellulose chromatography as described previously (16). After the addition of 0.5 M KPO4 to a final concentration of 1 mM, the high salt eluate from the phosphocellulose resin (550 ml, 1.1 g) was applied to a 2.6 × 40 cm hydroxylapatite column that had been pre-equilibrated with 50 mM Tris–HCl (pH 7.5), 0.75 M NaCl, 10 mM 2-mercaptoethanol, 1 mM KPO4. After washing with this buffer, proteins were eluted stepwise with 50 mM KPO4 (pH 7.5), 200 mM KPO4 (pH 7.5) and 400 mM KPO4 (pH 7.5) buffers, each containing 0.5 mM DTT. Fractions from each of the eluates that contained significant amounts of protein were pooled. Aliquots from these pools were dialyzed against 50 mM Tris–HCl (pH 7.5), 30 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 50% glycerol and assayed for DNA ligase activity.

The 400 mM KPO4 eluate (300 ml, 150 mg) was dialyzed against 50 mM Tris–HCl (pH 7.5), 30 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 10% glycerol (buffer A) and then loaded onto a 1.6 × 25 cm phosphocellulose column that had been pre-equilibrated with buffer A. Bound proteins were eluted with a 500 ml linear gradient from buffer A to buffer B containing 1 M NaCl and frations were assayed for DNA ligase activity. The DNA joining activity (48 ml, 15 mg, 11 U) was dialyzed against 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 0.5 mM DTT (buffer B) containing 0.9 M ammonium sulfate prior to loading onto a 15 ml phenyl–Sepharose column. Bound proteins were eluted with a 150 ml linear gradient from 0.9 to 0 M ammonium sulfate in buffer B and then with a 150 ml
linear gradient from buffer B to buffer containing 70% ethylene glycol. No DNA ligase activity was detected in the flow through, wash and gradient eluted fractions. DNA ligase activity (7 ml, 560 µg, 1.5 U) was eluted by buffer B containing 80% ethylene glycol.

Partial purification of yeast tRNA ligase

Yeast tRNA ligase was partially purified from 15 g (wet weight) E.coli SW1064 pKK223-3-RLGX cells by polymin P precipitation and heparin sepharose chromatography as described by Xu et al. (33). tRNA ligase activity was monitored by formation of the enzyme–AMP complex. The reactivity of the enzyme-adenylate intermediate formed by the peak fractions from the heparin–Sepharose column with different polynucleotide substrates and pyrophosphate was examined as described above.

Immunization procedures

Cdc9 protein, which was purified as described previously (16), was used as the antigen to produce a rabbit polyclonal antiserum by a standard immunization protocol (15).

Immunoprecipitation

Proteins were incubated with (5 µl) pre-immune serum or (5 µl) immune serum raised against Cdc9 protein for 90 min at 4°C in a final volume of 500 µl. Protein A–Sepharose beads, mixed with Sepharose CL-4B beads in a ratio of 1:3 (100 µl of a 50% suspension) were added and the incubation was continued for 60 min. The beads, which were collected by centrifugation and washed three times with lysis buffer, were resuspended in an equal volume of 60 mM Tris–HCl (pH 8.0), 10 mM MgCl₂, 5 mM DTT, 50 µg/ml bovine serum albumin and 2 mM sodium pyrophosphate. After incubation for 15 min at room temperature to remove AMP residues that had bound in vivo or during purification, the beads were washed with lysis buffer (16) and resuspended in an equal volume of SDS sample buffer. Labeled polypeptides were detected as described above.

Immunoblotting

Polypeptides were separated by denaturing gel electrophoresis and transferred to nitrocellulose membranes. The membrane was incubated with the primary antibody and the resultant antigen–antibody complexes were detected by enhanced chemiluminescence (35).

RESULTS

Saccharomyces cerevisiae extracts contain a DNA ligase that is active with the oligo (pdT)/poly (rA) substrate

Four biochemically distinct DNA ligase activities have been identified in mammalian cell extracts (3,4). DNA ligases II, III and IV can be distinguished from the enzyme that functions in DNA replication (DNA ligase I in mammals and Cdc9 in S.cerevisiae) by their ability to catalyze the joining of oligo (pdT) molecules hybridized to a poly (rA) template (3,4,16). Therefore, we examined extracts of S.cerevisiae for a DNA joining activity that was active with the oligo (pdT)/poly (rA) substrate.

In a previous study we observed co-purification of such an activity with Cdc9 DNA ligase activity during gel filtration chromatography (16). Prior to this molecular sieving step the yeast whole cell extract was fractionated by batch adsorption to and elution from P11 phosphocellulose column (16). Fractions were assayed for protein concentration and DNA ligase activity as described in Materials and Methods. (A) DNA ligase assays with the oligo (pdT)/poly (dA) substrate, filled-in squares. (B) DNA ligase assays with the oligo (pdT)/poly (rA) substrate, open squares. Protein concentration, filled-in circles. (C) Fractions (2 µl) were assayed for enzyme–AMP complex formation as described in Materials and Methods. The positions of 14C-labeled molecular mass standards (Amersham) are indicated on the left.
that eluted at ∼0.4 M NaCl was detected in assays with the oligo (pdT)/poly (dA) substrate (Fig. 1A). A polypeptide doublet with a molecular mass of ∼87 kDa that formed a labeled enzyme–adenylate complex co-eluted with this DNA joining activity (compare Fig. 1A and C). In contrast, assays with the oligo (pdT)/poly (rA) substrate (rA) substrate revealed a major peak of activity that eluted at ∼0.5 M NaCl (Fig. 1B). A polypeptide that reproducibly formed a slightly larger (90 kDa) labeled enzyme–adenylate complex co-eluted with this oligo (pdT)/poly (rA) joining activity (compare Fig. 1B and C). Separation of the major 87 kDa DNA joining activity (Cdc9 DNA ligase or DNA ligase I) from the minor (90 kDa) activity detected with the oligo (pdT)/poly (rA) substrate was also observed when the single peak of DNA joining activity from the gel filtration column (16) was applied to and gradient eluted from an S Sepharose column (data not shown).

**Partial purification and characterization of S. cerevisiae DNA ligase II**

Because of the extreme lability and relatively low levels of the oligo (pdT)/poly (rA) joining activity a purification scheme was developed that was more suited to the rapid processing of large quantities of yeast whole cell extracts. After lysis and batch adsorption to P11 phosphocellulose (16), proteins in the high salt eluate from the phosphocellulose resin were loaded directly onto a hydroxylapatite column. Consistent with previous observations, Cdc9 DNA ligase (yeast DNA ligase I) from the minor (90 kDa) activity detected with the oligo (pdT)/poly (rA) substrate was also observed when the single peak of DNA joining activity from the gel filtration column (16) was applied to and gradient eluted from an S Sepharose column (data not shown).

The eluates from the hydroxylapatite column were assayed for polypeptides that formed polypeptide–AMP complexes. As expected, a labeled polypeptide with a molecular mass of 87 kDa was detected in assays with the 200 mM KPO₄ eluate (Fig. 2A). Smaller quantities of a labeled polypeptide with a slightly reduced electrophoretic mobility, corresponding to a molecular mass of 90 kDa, were detected in assays with the 400 mM KPO₄ eluate (Fig. 2A). Labeled polypeptides that are authentic intermediates in DNA joining reactions will react with appropriate polynucleotide substrates, resulting in loss of the labeled AMP group. In agreement with our previous studies (16), incubation of the 87 kDa Cdc9 ligase enzyme–AMP complex formed by the 200 mM KPO₄ eluate also reacted with both pyrophosphate and oligo (pdT)/poly (rA) (Fig. 2C). However, in contrast to Cdc9 DNA ligase, the 90 kDa polypeptide–AMP complex formed by the 90 kDa polypeptide–AMP complex was stable in the presence of oligo (pdT)/poly (rA) (Fig. 2B). The 90 kDa polypeptide–AMP complex formed by the 400 mM KPO₄ eluate also reacted with both pyrophosphate and oligo (pdT)/poly (dA) (Fig. 2C). However, in contrast to Cdc9 DNA ligase, the 90 kDa polypeptide–AMP complex formed by the 90 kDa polypeptide–AMP complex was stable in the presence of oligo (pdT)/poly (rA) (data not shown). This activity is dependent on a duplex structure and is consistent with the joining of the oligo (pdT)/poly (rA) substrate by the 400 mM KPO₄ eluate.

*Saccharomyces cerevisiae* tRNA ligase forms an enzyme–adenylate reaction intermediate of a similar size to the DNA ligase–AMP complexes (33). To exclude the possibility that the putative DNA ligase in the 400 mM KPO₄ eluate was in fact tRNA ligase, yeast tRNA ligase was partially purified from an E. coli strain following its overexpression. The labeled tRNA ligase–AMP complex did not react with either oligo (pdT)/poly (dA) or oligo (pdT)/poly (rA) (data not shown). The DNA ligase activity in the 400 mM KPO₄ eluate was purified further by gradient elution from a phosphocellulose column. The oligo (pdT)/poly (rA) joining activity co-eluted with a polypeptide that formed a labeled 90 kDa polypeptide–AMP complex at ∼0.5 M NaCl (Fig. 3A and B). Small amounts of contaminating Cdc9 DNA ligase, detected in fraction 58 by formation of the labeled 87 kDa polypeptide–AMP complex (Fig. 3B) were resolved from the oligo (pdT)/poly (rA) joining activity. A total of 11 U of DNA ligase activity were recovered in

![Figure 2. Formation of labeled polypeptide–AMP complexes after fractionation by hydroxylapatite chromatography: reactivity of polypeptide–AMP intermediates.](image-url)
Figure 3. Purification of *S. cerevisiae* DNA ligase II by phosphocellulose and phenyl superose chromatography. After dialysis, proteins in the 400 mM KPO$_4$ eluate from the hydroxylapatite column were applied to a phosphocellulose column. Bound proteins were eluted with a linear gradient of increasing ionic strength as described in Materials and Methods. Fractions were assayed for protein concentration and DNA ligase activity. 

(A) DNA ligase assays with the oligo (pdT)/poly (rA) substrate, open squares. Protein concentration, filled-in circles. 

(B) Fractions (2 µl) were assayed for enzyme–AMP complex formation as described in Materials and Methods. The positions of 14 C-labeled molecular mass standards are indicated on the left. DNA ligase activity was purified further by phenyl–Sepharose chromatography as described in Materials and Methods. 

(C) Fractions were assayed for DNA joining with the oligo (pdT)/poly (rA) substrate, open squares. 

(D) Fractions (2 µl) were assayed for enzyme–AMP complex formation.

The peak fractions from the phosphocellulose column. Assuming a yield of 25–50% (based on the yields for similar fractionation steps in the Cdc9 purification scheme), the level of this activity is 5–10-fold lower than that measured for Cdc9 DNA ligase (16) and is consistent with estimates derived from measuring enzyme–AMP formation. The peak fractions of DNA joining activity from the phosphocellulose column were fractionated by phenyl superose chromatography. DNA ligase activity bound very tightly to the resin and was only eluted with 80% ethylene glycol. Once again oligo (pdT)/poly (rA) joining activity co-eluted with a polypeptide which formed a labeled 90 kDa polypeptide–AMP complex (Fig. 3 C and D). This activity has been designated *S. cerevisiae* DNA ligase II because it can be distinguished from Cdc9 DNA ligase by differences in chromatographic properties and in polynucleotide substrate specificity.

Analysis of the protein content of *S. cerevisiae* DNA ligase II fractions from the phenyl superose column by Coomassie blue staining after denaturing gel electrophoresis revealed the presence of at least 8–10 polypeptides. We identified polypeptides with molecular masses similar to that of the labeled enzyme–adenylate complex. However, none of these polypeptides co-eluted with DNA ligase activity (data not shown). Attempts at further purification of *S. cerevisiae* DNA ligase II using resins such as native DNA cellulose and Mono S consistently resulted in extremely low yields of enzyme activity, suggesting that this is a very labile enzyme.

Saccharomyces cerevisiae DNA ligase II activity is not affected by alterations in the level of Cdc9 DNA ligase

We considered the possibility that *S. cerevisiae* DNA ligase II may be a modified form of Cdc9 DNA ligase. If this were the case alterations of Cdc9 DNA ligase would be expected to affect DNA ligase II activity. To examine this issue we prepared whole cell extracts from the strain BJ2168 and from the same strain harboring a CDC9 overexpression plasmid, and fractionated these extracts by hydroxylapatite chromatography. As expected, when DNA ligase activity from the untransformed strain was monitored by enzyme–adenylate formation, radiolabeled 87 and 90 kDa complexes were detected in the 200 and 400 mM KPO$_4$ eluates respectively (Fig. 4 A). Overexpression of Cdc9 DNA ligase resulted in a large increase in the amount of the labeled 87 kDa complex (Fig. 4 B).
kDa DNA ligase complex in the 200 mM KPO₄ eluate (Fig. 4B). In contrast, the level of the labeled 90 kDa complex in the 400 mM KPO₄ eluate was not significantly affected (Fig. 4B).

The level of DNA ligase II activity was also examined in ade9 mutant background. Since DNA ligase activity is not detectable in whole cell extracts prepared from the strain L9-94D cdc9-7 grown at the permissive temperature (14), we compared the levels of DNA joining enzymes in fractionated extracts from this strain with identically fractionated extracts from the closely related CDC9+ strain M1-2B CDC9. Extracts from strains such as L9-94D and M1-2B contain wild-type levels of protease activity and hence yield lower levels of DNA ligase activity compared with extracts from protease-deficient strains such as BJ2168. We therefore utilized a more sensitive assay which employs a DNA substrate with a single defined nick that is more efficiently ligated and is less susceptible to nuclease degradation than the homopolymer substrates (16). Using this assay we were able to quantitate DNA joining activity in fractionated extracts containing wild-type levels of protease activity. As expected, Cdc9 DNA ligase activity was observed in the 200 mM KPO₄ eluate from the wild type strain M1-2B but not in the 200 mM KPO₄ eluate from the cdc9 strain L94-4D (Table 1). Furthermore, the 200 mM KPO₄ eluate from the wild-type strain contained an 87 kDa polypeptide that formed a labeled enzyme–AMP complex and was recognized by a Cdc9 antiserum, whereas this polypeptide was not detectable in the 200 mM KPO₄ eluate from the cdc9 strain (data not shown). In contrast, similar levels of DNA ligase activity (Table 1) and a 90 kDa polypeptide that formed a labeled enzyme–AMP complex (data not shown) were present in the 400 mM KPO₄ eluates from both the wild type and cdc9 strains. The observations that the level of the minor DNA ligase activity is not altered by either overexpression or inactivation of the CDC9 gene support our contention that this represents a second DNA ligase (DNA ligase II) in S. cerevisiae.

Table 1. Levels of Cdc9 DNA ligase (DNA ligase I) and DNA ligase II in fractionated extracts from wild type and cdc9 yeast strains

<table>
<thead>
<tr>
<th>Yeast strain</th>
<th>DNA ligase specific activity (U/mg)</th>
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<tr>
<td></td>
<td>200 mM KPO₄</td>
</tr>
<tr>
<td>M1-2B CDC9</td>
<td>0.1</td>
</tr>
<tr>
<td>L94-4D cdc9-7</td>
<td>&lt;0.01</td>
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Lyastes were prepared from M1-2B CDC9 and L94-4D cdc9-7 cells (~40 g of each) and fractionated by phosphocellulose and hydroxylapatite chromatography as described in Materials and Methods. After dialysis, the 200 and 400 mM KPO₄ eluates were assayed for DNA joining activity with the DNA substrate containing a single nick (16) as described in Materials and Methods. Protein concentrations were determined by the method of Bradford (36).

DNA ligase II is not recognized by antisera against Cdc9 DNA ligase

To provide further evidence that Cdc9 DNA ligase (DNA ligase I) and DNA ligase II are the products of distinct genes we examined the immunoreactivity of the polypeptides in the 200 and 400 mM KPO₄ hydroxylapatite fractions that formed enzyme–AMP complexes (Fig. 2) with a polyclonal Cdc9 antiserum. This antiserum immunoprecipitated a single polypeptide that formed an 87 kDa enzyme–AMP complex from yeast whole cell extracts (Fig. 5A). In similar experiments the Cdc9 polypeptide in the 200 mM KPO₄ eluate was also specifically immunoprecipitated and then adenylated (Fig. 5B, lane 3). However, no polypeptides were adenylated in the immunoprecipitate from the 400 mM KPO₄ eluate (Fig. 5B, lane 5). To exclude the possibility that the 400 mM KPO₄ eluate contains an activity which inhibits specific immunoprecipitation and/or adenylylation, we preincubated mixtures of the 200 and 400 mM KPO₄ eluates which by themselves yielded similar amounts of labeled polypeptide prior to immunoprecipitation. A single labeled 87 kDa polypeptide corresponding in size and quantity to that expected from the 200 mM KPO₄ eluate was detected (data not shown).

The hydroxylapatite fractions (Fig. 2) and the more purified fractions of DNA ligase II from the phenyl–Sepharose column were examined for Cdc9 protein by immunoblotting. As expected, Cdc9 was present in the 200 mM KPO₄ fraction (Fig. 6A, lane 1). A reactive polypeptide was also present in the 400 mM KPO₄ fraction (Fig. 6A, lane 2), but the level of this polypeptide was much lower compared with the 200 mM KPO₄ fraction. The absence of a signal in the more highly purified fractions of S. cerevisiae DNA ligase II (Fig. 6A, lane 3) suggests that the weak signal in the 400 mM KPO₄ fraction is due to contaminating Cdc9 protein and that the Cdc9 antibody does not cross-react with DNA ligase II. Similar results were obtained with a peptide antiserum that recognizes Cdc9 (16) and mammalian DNA ligases I, II and III (6,15) (Fig. 6B). This peptide antiserum was raised against a sequence that is highly conserved in all eukaryotic DNA ligases (5,8,14). The apparent lack of recognition of S. cerevisiae DNA ligase II by this reagent may be due to low levels of the DNA ligase II polypeptide in the fractions examined or the absence of the epitope recognized by the peptide antiserum. In this regard it is noteworthy that this peptide sequence is poorly conserved in the yeast open reading frame YOR005c that may encode a DNA ligase (see Discussion).

Figure 5. Immunoprecipitation of Cdc9 DNA ligase and DNA ligase II with Cdc9 antiserum. (A) BJ2168 cells (50 ml culture, OD₆₀₀~1.6) were harvested, resuspended in 1 ml of lysis buffer (16) and lysed by mechanical shearing with glass beads. Aliquots of the crude lysate (100 µl) were diluted to a final volume of 500 µl with lysis buffer and then incubated with; lane 2, pre-immune serum; lane 3, Cdc9 antiserum. (B) Aliquots (50 µl, 35 µg) of the 200 mM KPO₄ eluate from the hydroxylapatite column (see Fig. 2) were incubated with; lane 2, pre-immune serum; lane 3, Cdc9 antiserum. Aliquots (50 µl, 25 µg) of the 400 mM KPO₄ eluate from the hydroxylapatite column (see Fig. 2) were incubated with; lane 4, pre-immune serum; lane 5, Cdc9 antiserum. Immunoprecipitations and incubation with [α-³²P]ATP were carried out as described in Materials and Methods. Polypeptides in the immunoprecipitates were separated by electrophoresis through a 7.5% SDS–PAGE. The gel was then fixed, dried and exposed to X-ray film.
DNA ligase III by a proteolytic mechanism (5–7), is also unable to define a DNA ligase as a type III or type IV enzyme because (dT) template (3), activity with this substrate cannot be used to define DNA ligase III to join oligo (rA) molecules hybridized to a poly (dT) template (phenyl- Sepharose fraction, see Fig. 3). The positions of molecular mass standards (BioRad) are shown on the left and the position of Cdc9 DNA ligase is indicated on the right. (A) Cdc9 antisemur. (B) Antiserum raised against 16 amino acid sequence that is conserved in eukaryotic DNA ligases (15).

DISCUSSION

We have presented evidence for the presence of two distinct species of DNA ligase in mitotic extracts of S.cerevisiae. One of these enzymes, encoded by the CDC9 gene, has previously been shown to be functionally homologous to mammalian DNA ligase I and therefore can be considered to be the DNA ligase I of S.cerevisiae (14,16). This type of DNA ligase is required for DNA replication (9,11,12,14,37,38) and also functions in DNA repair (12,13,37). A second minor DNA joining activity was resolved from Cdc9 DNA ligase by fractionation on several different chromatography resins. We have designated this enzyme S.cerevisiae DNA ligase II, since it appears to be distinct from Cdc9 DNA ligase based on the following observations: (i) unlike Cdc9 DNA ligase, this enzyme is active on an oligo (pdT)/oligo (rA) substrate; (ii) the level of S.cerevisiae DNA ligase II activity was unaffected by overexpression or mutational inactivation of the CDC9 gene; (iii) S.cerevisiae DNA ligase II was not recognized by Cdc9 antisemur.

The ability to join oligo (dT) molecules hybridized to a poly (rA) template is a characteristic feature of S.cerevisiae DNA ligase II and mammalian DNA ligases II, III and IV (3,4) and specifically distinguishes these enzyme activities from the enzymes that function in DNA replication such as mammalian DNA ligase I and Cdc9 DNA ligase (4,16). Although mammalian DNA ligases III and IV can be distinguished by the ability of DNA ligase III to join oligo (rA) molecules hybridized to a poly (dT) template (3), activity with this substrate cannot be used to define a DNA ligase as a type III or type IV enzyme because mammalian DNA ligase II, which appears to be derived from DNA ligase III by a proteolytic mechanism (5–7), is also unable to join oligo (rA) molecules hybridized to a poly (dT) template (4). Hence, we do not interpret the absence of joining activity with the oligo (rA)/poly (dT) substrate as evidence that yeast DNA ligase II is more similar to mammalian DNA ligase IV than to mammalian DNA ligase III.

The very low levels and lability of yeast DNA ligase II have hindered our attempts to purify and characterize this enzyme more thoroughly. We have not been able to isolate sufficient quantities of this protein from mitotic extracts for peptide sequencing, which in turn would facilitate gene cloning. However, the recent availability of the complete sequence of the S.cerevisiae genome offers an alternative approach to identifying genes that may encode DNA ligases. A six amino acid motif required for formation of the enzyme–adenylate intermediate (7,39) and a 16 amino acid sequence of unknown function (5,8,14,15) are highly conserved in all known eukaryotic DNA ligases. The latter sequence was used to identify human DNA ligase cDNAs encoding DNA ligases I, III and IV in a search of a human EST database (8), but does not identify open reading frames other than the one encoded by the CDC9 gene in the S.cerevisiae genome. In contrast, searches of the S.cerevisiae genome with the active site motif KYDGER identified the CDC9 gene as well as a second open reading frame designated YOR005c which encodes a 944 amino acid polypeptide. There are additional regions within this open reading frame that share less extensive homology with conserved regions (including the 16 amino acid sequence of unknown function) in the catalytic domains of other eukaryotic DNA ligases. The relationship between this putative DNA ligase, which most closely resembles human DNA ligase IV, and S.cerevisiae DNA ligase II is currently being investigated.

The relative ease of genetic manipulation in S.cerevisiae makes this organism an attractive model system for higher eukaryotes. For example, molecular and genetic analysis of the yeast genome has identified functional homologues for mammalian DNA polymerases α, β, 8 and ε (40–44). The identification of a yeast open reading frame that may encode a DNA ligase coupled with our biochemical demonstration of a yeast DNA ligase activity with similar properties to mammalian DNA ligases III and IV, suggests that genetic analysis of the multiple species of eukaryotic DNA ligase should be tractable in S.cerevisiae, and that the results of such studies will be informative with respect to DNA metabolism in mammalian cells.

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