DNA Ligase I from *Saccharomyces cerevisiae*: Physical and Biochemical Characterization of the *CDC9* Gene Product†

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Received June 29, 1992; Revised Manuscript Received September 2, 1992

ABSTRACT: Genetic studies have previously demonstrated that the *Saccharomyces cerevisiae CDC9* gene product, which is functionally homologous to mammalian DNA ligase I, is required for DNA replication and is also involved in DNA repair and genetic recombination. In the present study we have purified the yeast enzyme. When measured under denaturing conditions, Cdc9 protein has a polypeptide molecular mass of 87 kDa. The native form of the enzyme is an 80-kDa asymmetric monomer. Both estimates are in good agreement with the M_r = 84 406 predicted from the translated sequence of the *CDC9* gene. Cdc9 DNA ligase acts via the same basic reaction mechanism employed by all known ATP-dependent DNA ligases. The catalytic functions reside in a 70-kDa C-terminal domain that is conserved in mammalian DNA ligase I and in Cdc17 DNA ligase from *Schizosaccharomyces pombe*. The ATP analog ATPαS inhibits the ligation reaction, although Cdc9 protein does form an enzyme–thioadenylate intermediate. Since Cdc9 DNA ligase exhibited the same substrate specifity as mammalian DNA ligase I, this enzyme can be considered to be the DNA ligase I of *S. cerevisiae*. There is genetic evidence suggesting that DNA ligase may be directly involved in error-prone DNA repair. We examined the ability of Cdc9 DNA ligase to join nicks with mismatches at the termini. Mismatches at the 5′ termini of nicks had very little effect on ligation, whereas mismatches opposite a purine at 3′ termini inhibited DNA ligation. The joining of DNA molecules with mismatched termini by DNA ligase may be responsible for the generation of mutations.

DNA ligation is an essential step in DNA replication, DNA repair, and genetic recombination. Genetic and biochemical studies in bacteria and yeast suggest that this reaction is catalyzed by single species of DNA ligase (Kornberg & Baker, 1991). In contrast, two and three distinct species of DNA ligase have been purified and characterized from *Drosophila melanogaster* cells (Takahashi & Tomizawa, 1990) and from mammalian tissues and cells (Tomkinson et al., 1991a; Lindahl & Barnes, 1992), respectively.

Mutants in the *CDC9* and *cdc17*$^+$ DNA ligase genes of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* were originally isolated in screens for conditional lethal mutants that arrest during cell division (Hartwell et al., 1973; Nasmyth, 1977). These mutants have a similar phenotype to DNA ligase mutants of *Escherichia coli*. They accumulate Okazaki fragments during DNA replication, exhibit enhanced mitotic recombination, and are sensitive to many different types of DNA damage (Johnston & Nasmyth, 1978; Fabre & Roman, 1979; Johnston, 1979). Both *S. cerevisiae cdc9* and *Sch. pombe cdc17* mutant cells grown at the permissive temperature contain a reduced level of a temperature-sensitive DNA ligase activity, confirming that these genes encode DNA ligases (Nasmyth, 1979; Barker et al., 1985).

The *CDC9* gene of *S. cerevisiae* and the *cdc17*$^+$ gene of *Sch. pombe* have been cloned and sequenced (Barker & Johnston, 1983; Barker et al., 1983, 1987; Johnston et al., 1986). The amino acid sequences of the predicted proteins share 53% identity, with the homology confined to the C-terminal regions (Barker et al., 1987). Transcription of the *CDC9* gene is cell cycle-regulated and is also induced by DNA damage, whereas expression of the *cdc17*$^+$ gene is not regulated at the level of transcription during the cell cycle (Barker et al., 1983, 1987; Peterson et al., 1985; White et al., 1986).

The human DNA ligase I gene also encodes a protein with extensive amino acid identity with the translated *S. cerevisiae CDC9* gene (Barnes et al., 1990). Human DNA ligase I cDNA complements the conditional lethal phenotype of a *S. cerevisiae cdc9* mutant (Barnes et al., 1990), as well as the altered recombination and DNA repair-defective phenotypes of this mutant.[1] These observations suggest that human DNA ligase I and yeast Cdc9 protein are functionally homologous.

The functional roles of mammalian DNA ligases II and III in living cells cannot be predicted solely from their biochemical properties in vitro. Thus, we have begun to systematically examine the multiplicity of DNA ligases in the yeast *S. cerevisiae*, a eukaryotic organism that is highly amenable to genetic analysis. Although the *CDC9* gene has been extensively studied by genetic and molecular techniques, the biochemical properties of *S. cerevisiae* (Cdc9) DNA ligase I have not been documented. In this report we describe the purification of Cdc9 protein from *S. cerevisiae* to >95% homogeneity and the characterization of its DNA ligase activity.

EXPERIMENTAL PROCEDURES

Plasmids and Strains. A plasmid containing the *CDC9* gene, p207Sclig1BH (Banks & Barker, 1985), was provided by Dr. L. H. Johnston, Laboratory of Cell Propagation, National Institute of Medical Research, U.K. The *S. cerevisiae* strain, BJ2168 (*MATα prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52*), was from the Yeast Genetic Stock Center,

† These studies were supported by U.S. Public Health Service Grant CA12428 from the National Cancer Institute, Department of Health and Human Services.

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[1] L. H. Johnston, personal communication.

0006-2960/92/0431-11762$03.00/0

Berkeley, CA. After preparation by the alkaline lysis method (Sambrook et al., 1989), plasmid p207Sclig1BH was transformed into strain BJ2168 by the spheroplast method, selecting for growth on synthetic complete medium minus leucine (Burgers & Percival, 1987).

Strain BJ2168 (p207Sclig1BH) was grown in yeast minimal media [1.7 g of yeast nitrogen base/L, 20 g of dextrose/L, 5 g of $(NH_4)_2SO_4$] supplemented with 20 mg/L uracil and 40 mg/L tryptophan. The strain was grown on a large scale in a 60-L New Brunswick Fermentor at 30 °C, pH 5.5. Approximately 350 g of cells (wet weight) was obtained from 59 L of culture.

Preparation of Homopolymer Polynucleotide Substrates. Oligonucleotides $(pdT)_{16}$, $(dT)_{16}$, and $(rA)_{12-18}$ and polynucleotides poly(dA), poly(rA), and poly(dT) were purchased from Pharmacia. Dephosphorylated oligonucleotides (10 μg, reaction volume 50 μL) were labeled with 100 μCi of [γ-^{32}P]-ATP (3000 Ci/mmol, Amersham), with cold ATP added to a final concentration of 2 μM, using T4 polynucleotide kinase (New England Biolabs). Labeled oligonucleotides were purified by centrifugation through a Sephadex G-25 (Pharmacia) column. Oligonucleotides and polynucleotides were mixed in equimolar amounts, incubated at 90 °C for 10 min, and then slowly cooled to room temperature.

Preparation of Polynucleotide Substrate with a Defined Single Nick or Gap. Oligonucleotides of defined sequence (Table II) were synthesized on an Applied Biosystems Model 394 DNA/RNA synthesizer. These oligonucleotides, which have 5′- and 3′-hydroxyl termini, were end-labeled as described above and examined following separation through a 20% denaturing polyacrylamide gel. A derivative of oligo #2 with a 3′-dideoxy adenine residue was produced by hybridizing oligo #1 lacking the 3′-nucleotide with oligo #3. A 3′-dideoxyadenosine residue was added by incubation in the presence of the Klenow fragment of *Escherichia coli* DNA polymerase I and 0.4 mM ddATP as described by Sambrook et al. (1989). The oligonucleotides were purified by centrifugation through a Sephadex G-25 (Pharmacia) column. An aliquot was taken, heat-denatured, and end-labeled. Conversion of greater than 90% of the 17-mer to a form with the same mobility as full-length oligo #1 was determined as described above.

^{32}P-Labeled oligo #2 was hybridized with oligo #1 and oligo #3 as described above. Several variations of this substrate were constructed with the following derivatives of oligo #1: oligo #1 with a 3′-dideoxy adenine to generate a nick with a 5′-phosphoryl terminus but no 3′-hydroxyl; oligo #1 with 1- and 2-base 3′ deletions to generate a substrates with 1- and 2-nucleotide gaps; oligo #1 with either a 3′ cytosine or 3′ guanine replacing the 3′ adenine to generate a nick with mismatched 3′ termini, and with the following ^{32}P-labeled derivatives of oligo #2: oligo #2 with either 5′ adenine or 5′ thymine replacing the 5′ guanine to generate a nick with mismatched 5′ termini.

^{32}P-Labeled oligo #5 was hybridized with oligo #4 and oligo #6 as described previously. Several variations of this substrate were constructed with the following derivatives of oligo #4: oligo #4 with either a 3′ adenine or 3′ thymine replacing the 3′ cytosine to generate a nick with mismatched 3′ termini, and with the following ^{32}P-labeled derivatives of oligo #5: oligo #5 with either 5′ cytosine or 5′ guanine replacing the 5′ thymine to generate a nick with mismatched 5′ termini.

DNA Ligase Assays. Reactions were carried out as described by Tomkinson et al. (1990). In certain assays, ATP was replaced with 1 mM ATPαS.[2] One unit of DNA ligase activity catalyzes the conversion of 1 nmol of terminal phosphate residues to a phosphatase-resistant form in 15 min at 20 °C.

Formation of DNA Ligase–Adenylate Complex. Reactions were carried out as described by Tomkinson et al. (1990). In certain assays, [α-^{32}P]ATP was replaced with [^{35}S]ATPαS (1000 Ci/mmol, Amersham). Incubations were at room temperature for 15 min. After the addition of 5 μL of SDS sample buffer, reaction mixtures were heated at 90 °C for 10 min. Proteins were separated by SDS–PAGE[2] (Laemmli, 1970). Gels were fixed for 10 min in 10% acetic acid and dried, and adenylylated proteins were detected by autoradiography.

To examine the reactivity of the enzyme–adenylate intermediate, the adenylylation reaction was performed as described above in a final volume of 40 μL. Aliquots (10 μL) were incubated either with 0.8 μg of unlabeled polynucleotide substrate or with 20 nmol of sodium pyrophosphate for 2 h at 20 °C. Reactions were stopped by the addition of SDS sample buffer, and adenylylated polypeptides were detected as described previously.

Formation of DNA–Adenylate Intermediate. Reaction mixtures (40 μL) containing 60 mM Mes[2]–NaOH (pH 6.4), 10 mM $MgCl_2$, 5 mM DTT, 50 μg/mL bovine serum albumin, 20 μCi [α-^{32}P]ATP (3000 Ci/mmol, Amersham), and Cdc9 protein were incubated for 15 min at room temperature. In certain assays, [α-^{32}P]ATP was replaced with [^{35}S]ATPαS (1000 Ci/mmol, Amersham). After the addition of polynucleotide substrate (5 μg), incubation was continued at either 0 or 20 °C. Aliquots (5 μL) were removed at various times and added to 2 μL of 50 mM EDTA (pH 8.0). Formamide dye (2 μL) was added to the samples, which were then heated at 80 °C for 2 min prior to loading (2 μL) onto a 20% denaturing polyacrylamide gel. After electrophoresis for 2 h at 70 W, gels were dried and labeled oligonucleotides were detected by autoradiography.

Analysis of Ligation Products. DNA ligase assays were carried out as described previously (Tomkinson et al., 1990). An aliquot (10 μL) was added to 10 μL of formamide dye and heated for 2 min at 80 °C. Samples (2.5 μL) were loaded onto a denaturing 20% polyacrylamide gel. After electrophoresis for 2 h at 70 W, gels were dried and oligonucleotides were visualized by autoradiography.

Analytical Gel Filtration and Glycerol Density Gradient Sedimentation. Gel filtration was carried out on an FPLC[2] Superose 12 column as described by Tomkinson et al. (1990). The Stokes radius of Cdc9 protein was calculated according to Siegel and Monty (1966).

Cdc9 protein (fraction VII) was sedimented through an 11-mL 10–30% glycerol gradient in 50 mM Tris-HCl (pH 7.5), 1 M NaCl, 1 mM EDTA, and 0.5 mM DTT. Gradients were centrifuged at 40 000 rpm in a Beckman SW40.1 rotor for 40 h at 4 °C. Fractions were collected from the bottom of the tube, and Cdc9 protein was detected by the formation of polypeptide–AMP complex. [*methyl*-^{14}C]Bovine serum albumin ($4.4S_{20,w}$) (Amersham) was used as an internal molecular mass standard and was also centrifuged in a separate tube with γ-globulin ($7.3S_{20,w}$), ovalbumin ($3.66S_{20,w}$), and

[2] Abbreviations: ATPαS, adenosine 5′-α-thiotriphosphate; DTT, dithiothreitol; FPLC, fast protein liquid chromatography; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone.

myoglobin ($1.97S_{20,w}$). The $S_{20,w}$ value for Cdc9 protein was calculated according to Martin and Ames (1961).

Immunoblots. Proteins were separated by SDS–PAGE and transferred to nitrocellulose membranes (Schleicher and Schuell). Antigen–antibody complexes were detected as described by Tomkinson et al. (1990).

Amino Acid Sequencing. Proteins were separated by SDS–PAGE[2] according to the method of Hunkapillar et al. (1983) and then transferred to poly(vinylidene fluoride) membranes (Millipore) as described by Matsudaira (1987). N-Terminal amino acid sequencing was performed on an Applied Biosystems Model 477A liquid-phase sequencer coupled to an on-line Model 120A high-performance liquid chromatograph. In the absence of amino acid sequence, the apparently blocked polypeptide was cleaved by incubation with cyanogen bromide and the N-terminal amino acid sequences of the mixture of cyanogen bromide-peptides were determined as described above.

Purification of Cdc9 Protein. The cell pellet (180 g) was thawed and resuspended in 50 mM Tris-HCl (pH 7.5), 0.4 M NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM PMSF,[2] 1 mM benzamidine hydrochloride, 5 μg/mL chymostatin, leupeptin, and TLCK,[2] 2.5 μg/mL pepstatin, and 1.9 μg/mL aprotinin (protease inhibitors from Sigma) at room temperature. All subsequent steps were carried out on ice or at 4 °C. The cells were disrupted by homogenization with zirconium beads (0.5 mm) in a Biospec Bead Beater for 6 pulses of 30 s, with a 2-min interval between each pulse to allow for cooling. Insoluble cellular debris was removed by centrifugation for 45 min at 45 000 rpm in a Ti-70 rotor (Beckman Instruments). The cleared lysate (fraction I, 320 mL) was diluted 20-fold with 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM 2-mercaptoethanol, and batch adsorbed to a 1.5-L-thick slurry of P11 phosphocellulose (Whatman) that had been equilibrated with 50 mM Tris-HCl (pH 7.5), 20 mM NaCl, 1 mM EDTA, and 10 mM 2-mercaptoethanol (buffer A). The resin was washed with 10 L of buffer A containing the following protease inhibitors: 1 mM PMSF, 1 mM benzamidine hydrochloride, 0.5 μg/mL chymostatin, leupeptin, and TLCK, 0.25 μg/mL pepstatin, and 0.19 μg/mL aprotinin. Unless otherwise stated all subsequent buffers contain protease inhibitors at the concentration described above. The P11 phosphocellulose was poured into two columns of 5 × 35 cm. Proteins were eluted with buffer A containing 0.7 M NaCl (fraction II, 470 mL).

Ammonium sulfate (0.194 g/mL) was added slowly, and a neutral pH was maintained by the addition of 1 M Tris base. After stirring for 30 min, precipitate was removed by centrifugation at 10 000 rpm for 30 min in a JA-10 rotor (Beckman Instruments), and ammonium sulfate (0.201 g/mL) was added to the supernatant. The precipitate was collected by centrifugation as described previously and resuspended in 50 mM Tris-HCl (pH 7.5), 1 M NaCl, 1 mM EDTA, and 0.5 mM DTT (buffer B). Proteins were dialyzed for 4 h against buffer B. After centrifugation for 20 min at 10 000 rpm in a JA-20 rotor (Beckman Instruments), the dialysate (fraction III, 22 mL) was applied to a 2.6- × 84-cm Ultrogel AcA 44 (IBF Biotechnics) column, which had been preequilibrated with buffer B. Proteins were eluted with buffer B, and fractions containing DNA ligase activity were pooled (fraction IV, 52 mL). After the addition of 0.5 M KPO_4 (pH 7.5) to a final concentration of 1 mM, fraction IV was applied to 2.6- × 20-cm hydroxylapatite (Bio-Rad) column, which had been preequilibrated with 50 mM Tris-HCl (pH 7.5), 1 M NaCl, 1 mM KPO_4, and 0.5 mM DTT. After washing with this buffer, proteins were eluted stepwise with 50 mM KPO_4 (pH 7.5), 200 mM KPO_4 (pH 7.5), and 400 mM KPO_4 (pH 7.5) buffers, each containing 0.5 mM DTT. DNA ligase activity, which was eluted by the 200 mM KPO_4 buffer, was dialyzed against 50 mM Tris-HCl (pH 7.5), 30 mM NaCl, 1 mM EDTA, 0.5 mM DTT, and 10% glycerol (buffer C).

Table I: Purification of Cdc9 DNA Ligase from *S. cerevisiae*[a]

fraction	protein (mg)	total activity (units)	specific activity (units/mg)
(I) crude extract	4400	(18)	(0.004)
(II) phosphocellulose	890	ND	ND
(III) ammonium sulfate	550	7.9	0.014
(IV) gel filtration	250	10.0	0.04
(V) hydroxylapatite	95	5.3	0.056
(VI) DNA–cellulose	15	1.9	0.13
(VII) FPLC Mono Q	2.0	1.8	0.9

[a] Purification of Cdc9 protein was carried out as described in Experimental Procedures. Protein concentrations were determined by the method of Bradford (1976). Cdc9 DNA ligase activity was measured quantitatively by the ligation assay and also by enzyme–adenylate formation. It was not possible to accurately measure DNA ligase activity in fractions I and II due to the presence of interfering enzyme activities and nucleic acids. The removal of these factors is illustrated by the apparent increase in total enzyme activity reproducibly observed following gel filtration. The values in parentheses for fraction I were estimated by immunoblotting, using an antiserum raised against a C-terminal epitope of Cdc9 protein (Tomkinson et al., 1990).

Fraction V (150 mL) was applied to a 1.6- × 20-cm double-stranded DNA cellulose column (Sigma). DNA ligase activity was eluted with buffer C containing 0.3 M NaCl but no protease inhibitors (fraction VI, 30 mL). Fraction VI was divided in half and further purified by two identical fractionations on a FPLC Mono Q HR 5/5 column (Pharmacia). After application to the Mono Q column, bound proteins were eluted with a 30-mL linear gradient from buffer C (no protease inhibitors) to buffer C containing 0.75 M NaCl. DNA ligase activity (fraction VII, 3 mL) eluted at 0.3 M NaCl and was either stored on ice or at −20 °C after dialysis against buffer C (no protease inhibitors) containing 50% glycerol or on ice. The fractions stored on ice were stable for at least 4 weeks, and the fractions stored at −20 °C were stable for several months.

RESULTS

Purification and Physical Characterization of S. cerevisiae Cdc9 DNA Ligase. Since Cdc9 DNA ligase and mammalian DNA ligase I share extensive amino acid homology, it was anticipated that these enzymes would have similar chromatographic properties. The scheme for the purification of the yeast enzyme is essentially the same as that used for calf thymus DNA ligase I (Tomkinson et al., 1990) and is summarized in Table I. The purification of Cdc9 DNA ligase was greatly facilitated by the use of the protease-defective yeast strain BJ2168, and by transformation of this strain with plasmid p207Sclig1BH, which resulted in an ~100-fold increase in the level of DNA ligase activity (Banks & Barker, 1985; data not shown).

Hydroxylapatite chromatography did not result in a significant increase in specific activity of Cdc9 DNA ligase. However, this step resolved the enzyme from another DNA ligase activity that copurified with Cdc9 protein in the gel filtration step.[3]

[3] A. E. Tomkinson, N. J. Tappe, and E. C. Friedberg, unpublished observations.

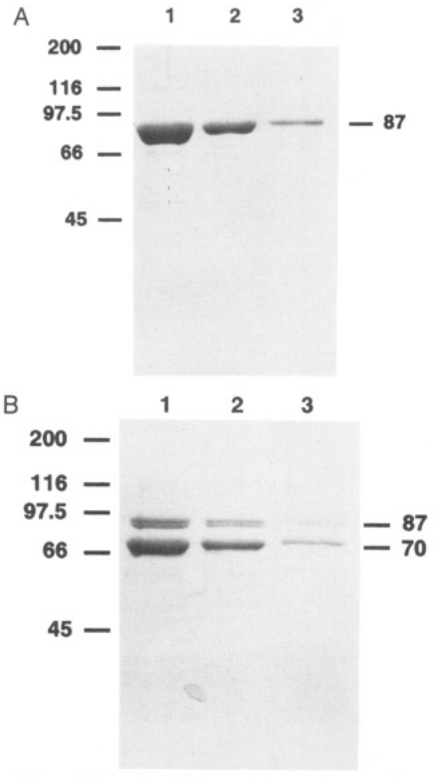

FIGURE 1: Size of intact Cdc9 protein and an active proteolytic fragment as measured by SDS–PAGE. (A) Cdc9 protein (fraction VII). Lane 1, 1.8 μg; lane 2, 0.6 μg; and lane 3, 0.2 μg. (B) Active proteolytic fragment of Cdc9 protein. Lane 1, 1.8 μg; lane 2, 0.6 μg; and lane 3, 0.2 μg. Molecular mass standards were as follows: myosin, 200 kDa; β-galactosidase, 116 kDa; phosphorylase *b*, 97.5 kDa; bovine serum albumin, 66 kDa; and ovalbumin, 45 kDa (Bio-Rad). After electrophoresis through a 10% denaturing acrylamide gel (Laemmli, 1970), proteins were detected by staining with Coomassie blue.

DNA ligase activity (fraction VII) coeluted with an 87-kDa polypeptide during Mono Q chromatography (Figure 1A). Additionally, incubation of fraction VII with [α-^{32}P]-ATP resulted in the formation of an 87-kDa enzyme–adenylate intermediate (Figure 2A, lane 2). Despite the conservation of amino acid sequence between yeast and human proteins, polyclonal antiserum against bovine DNA ligase I (Tomkinson et al., 1990) did not cross-react with Cdc9 protein (data not shown). However, rabbit polyclonal antiserum raised against a C-terminal epitope in bovine DNA ligase I (Tomkinson et al., 1990) cross-reacted with the 87-kDa band in fraction VII (Figure 3, lane 3). This epitope is conserved in the translated amino acid sequences of human DNA ligase I cDNA, the *S. cerevisiae CDC9* gene and the *Sch. pombe cdc17*$^{+}$ gene (Barker et al., 1983, 1987; Barnes et al., 1990). Collectively these results indicate that Cdc9 DNA ligase is a polypeptide with a molecular mass of 87 kDa. This estimate is in good agreement with the M_r of 84 406 predicted from the DNA sequence of the *CDC9* gene (Barker et al., 1983).

It was not possible to accurately measure DNA ligase activity in the crude extract (fraction I). However, we estimated the amount of Cdc9 protein in this fraction by immunoblotting with antiserum raised against the conserved C-terminal peptide (Tomkinson et al., 1990) and quantitating its reaction with homogeneous Cdc9 protein (fraction VII, Figure 3). The final yield of Cdc9 protein was calculated at 10%, and the enzyme was purified ~225-fold. This predicts the presence of about 150 000 Cdc9 protein molecules/cell. Since the plasmid p207Sclig1BH results in a 100-fold increase in the level of Cdc9 protein per cell, we estimate that an untransformed yeast cell contains ~1500 molecules of Cdc9 protein.

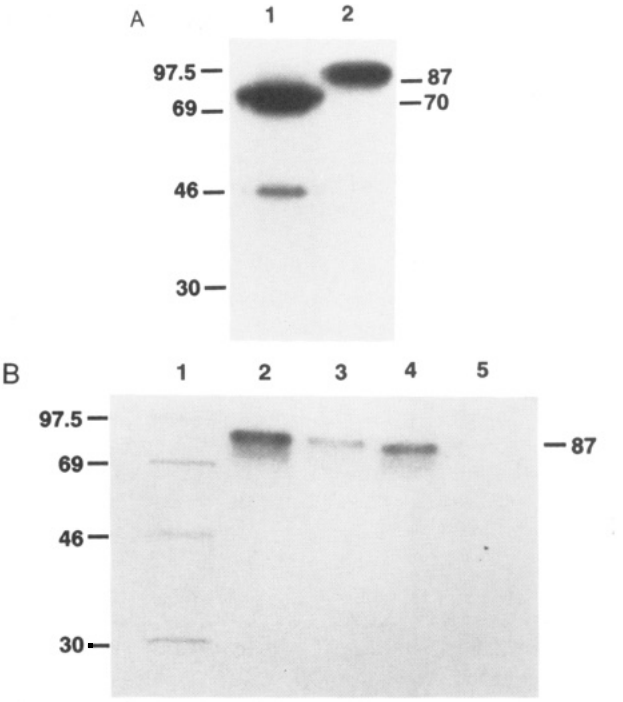

FIGURE 2: Adenylylation of intact Cdc9 protein and an active proteolytic fragment; reactivity of the enzyme–adenylate complex. Adenylylation reactions were carried out as described in Experimental Procedures. (A) Lane 1, 120 ng of Cdc9 active proteolytic fragment; lane 2, 120 ng of intact Cdc9 protein (fraction VII). (B) Lane 1, ^{14}C-labeled molecular mass standards: myosin, 200 kDa; phosphorylase *b*, 97.5 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa (Amersham). Cdc9–adenylate (fraction VII, 60 ng) was incubated with the following: lane 2, no addition; lane 3, oligo(pdT)·poly (dA); lane 4, oligo(pdT)·poly(rA); and lane 5, sodium pyrophosphate. Reactions were stopped by the addition of SDS sample buffer. Polypeptides were separated by electrophoresis through a 10% SDS–polyacrylamide gel, which was then fixed, dried, and exposed to X-ray film.

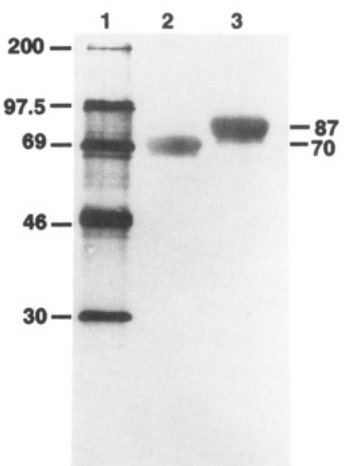

FIGURE 3: Antiserum raised against a C-terminal peptide common to several eukaryotic DNA ligases cross-reacts with intact Cdc9 protein and the active proteolytic fragment. Proteins were separated by electrophoresis through a 10% SDS–polyacrylamide gel and then transferred to a nitrocellulose membrane, which was incubated with a 1/1000 dilution of rabbit antiserum raised against the conserved peptide sequence (30). Antigen–antibody complexes were detected as described in the text. Lane 1, ^{14}C-labeled molecular mass standards as for Figure 2; lane 2, 1.2 μg of intact Cdc9 protein (fraction VII); and lane 3, 1.2 μg of active proteolytic fragment.

Purification of overexpressed Cdc9 protein from wild-type strains or from the protease mutant BJ2168 in the absence

of protease inhibitors resulted in the appearance of a doublet at 87 kDa and a smaller polypeptide with a molecular mass of 70 kDa (Figure 1B). Homogeneous preparations of Cdc9 protein (fraction VII) also converted to a doublet after storage for several weeks on ice. Both bands of the doublet formed an enzyme–adenylate complex and cross-reacted with the antiserum raised against the conserved C-terminal peptide (data not shown). The N-termini of both polypeptides in the doublet were blocked to Edman degradation chemistry. However, the results of N-terminal amino acid sequencing following cyanogen bromide cleavage indicated that the two polypeptides are closely related and were consistent with the predicted amino acid sequence of the *CDC9* gene. It is probable that the doublet is generated by limited proteolysis at the C-terminus of the protein, but we cannot exclude the possibility that the presence of two polypeptides reflects posttranslational modification. The relative distribution of the bands in the doublet was not altered by incubation with potato acid phosphatase, bacterial alkaline phosphatase, or 1 mM sodium pyrophosphate (data not shown).

The N-terminal amino acid sequence of the 70-kDa fragment is Ser-Ser-Ile-Pro, which corresponds to residues 106–109 in the predicted amino acid sequence of the *CDC9* gene (Barker et al., 1983). Hence, the N-terminal 105 amino acids were presumably removed by proteolysis. The 70-kDa polypeptide also cross-reacted with the antiserum raised against the conserved C-terminal peptide sequence (Figure 3, lane 2). Additionally, fractions containing the 70-kDa polypeptide as 80–90% of the total protein formed a 70-kDa polypeptide–AMP complex (Figure 2A, lane 1) and had a specific activity in the ligation assay similar to that of fraction VII (data not shown). We therefore conclude that the 70-kDa proteolytic fragment is the C-terminal catalytic domain of Cdc9 DNA ligase.

Hydrodynamic Properties of Native Cdc9 DNA Ligase. Cdc9 protein has a sedimentation coefficient of $4.4S_{20,w}$, which corresponds to a molecular mass of 69 kDa assuming a globular configuration. In gel filtration experiments Cdc9 protein has a Stokes radius of 43 Å, which corresponds to a molecular mass of 120 kDa, assuming a globular structure. Estimates of native molecular mass and frictional coefficient can be calculated from the sedimentation coefficient and Stokes radius values (Siegel & Monty, 1966), assuming a partial specific volume of 0.73 g/mL. Using these equations, Cdc9 protein has a predicted molecular mass of 80 kDa and a frictional coefficient of 1.5, indicating that the native enzyme is a monomer with a markedly asymmetric shape.

Properties of the Cdc9 DNA Ligase–Adenylate Intermediate. In the first step of the ligation reaction all viral and eukaryotic DNA ligases interact with ATP to form a covalent enzyme–adenylate intermediate. Both intact Cdc9 protein and the 70-kDa proteolytic fragment formed polypeptide–AMP complexes (Figure 2A). This complex is an authentic reaction intermediate since the AMP group was released by incubation with polynucleotide substrate such as oligo(pdT)$_{16}$ hybridized to poly(dA) (Figure 2B, lane 3) or by incubation with pyrophosphate, which reverses the adenylylation reaction (Figure 2B, lane 5). The Cdc9 DNA ligase–AMP complex was insensitive to incubation with oligo(pdT)$_{16}$ hybridized to poly(rA) (Figure 2B, lane 4), suggesting that this is not a suitable substrate for the next step of the ligation (see later).

Incubation of fraction VII with polynucleotide substrate in the absence of ATP indicated that ~2% of the Cdc9 protein molecules were adenylylated. This may not accurately reflect the in vivo level, since the enzyme was exposed to ATP and nicked DNA in the crude lysate and to nicked DNA during DNA cellulose chromatography.

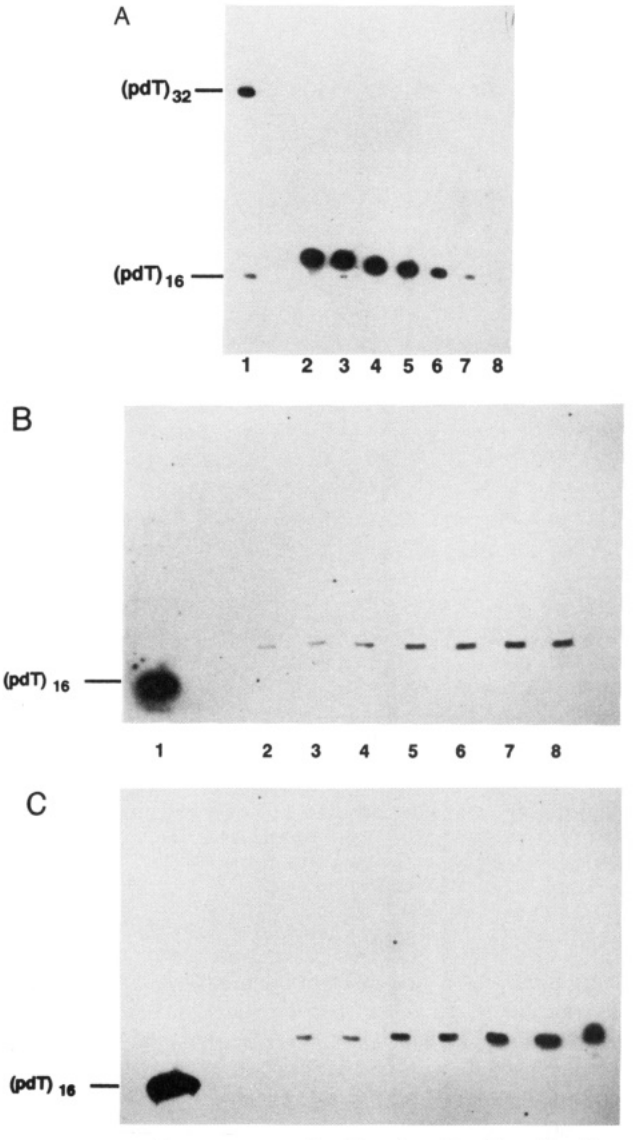

FIGURE 4: Formation of the DNA–adenylate and DNA–thioadenylate intermediate. The reaction of Cdc9–[^{32}P]adenylate or Cdc9–[^{35}S]adenylate with polynucleotide substrate was carried out as described in Experimental Procedures. (A) Lane 1, ^{32}P-labeled molecular mass standards. Incubation of Cdc9–[^{32}P]adenylate at 20 °C for the following times: lane 2, 15 s; lane 3, 30 s; lane 4, 45 s; lane 5, 1 min; lane 6, 2 min; lane 7, 5 min; lane 8, 10 min. (B) Lane 1, ^{32}P-labeled molecular mass standard. Incubation of Cdc9–[^{32}P]adenylate at 0 °C for the following times: lane 2, 5 min; lane 3, 10 min; lane 4, 15 min; lane 5, 20 min; lane 6, 30 min; lane 7, 45 min; lane 8, 60 min. (C) Lane 1, ^{32}P-labeled molecular mass standard. Incubation of Cdc9–[^{35}S]adenylate at 20 °C for the following times: lane 2, 5 min; lane 3, 10 min; lane 4, 15 min; lane 5, 30 min; lane 6, 45 min; lane 7, 60 min; lane 8, 90 min.

DNA–Adenylate Intermediate. In the next step of the ligation reaction the AMP group is transferred from the enzyme to the 5′-phosphoryl residue at a nick in double-stranded DNA. The pyrophosphate bond between the 5′-phosphoryl residue and the AMP moiety provides the energy for subsequent DNA ligase-catalyzed phosphodiester bond formation with the concomitant release of AMP. Cdc9 protein–[^{32}P]AMP was incubated with 5′-phosphorylated oligo(dT)$_{16}$ hybridized to poly(dA). The [^{32}P]AMP group was transferred from the enzyme to the oligonucleotide, generating adenylylated oligo(pdT)$_{16}$ (Figure 4A). At 20 °C

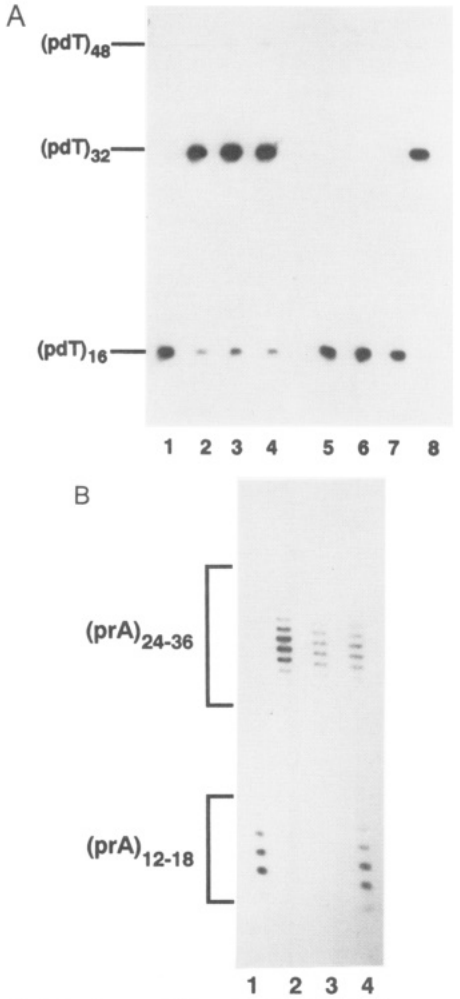

FIGURE 5: Ligation activity of Cdc9 DNA ligase on different homopolymer substrates. Reactions were carried out as described in Experimental Procedures. (A) Lanes 1–4, ligation of oligo-(pdT)·poly (dA) by the following: lane 1, no addition; lane 2, 180 ng of Cdc9 DNA ligase (fraction VII); lane 3, 180 ng of Cdc9 proteolytic fragment; and lane 4, 0.3 unit of T4 DNA ligase (BRL). Lanes 5–8, ligation of oligo(pdT)·poly(rA) by the following: lane 5, no addition; lane 6, 180 ng of Cdc9 DNA ligase (fraction VII); lane 7, 180 ng of Cdc9 proteolytic fragment; and lane 8, 0.3 unit of T4 DNA ligase (BRL). (B) Ligation of oligo(prA)·poly(dT) by the following: lane 1, no addition; lane 2, 180 ng of Cdc9 DNA ligase (fraction VII); lane 3, 180 ng of Cdc9 proteolytic fragment; and lane 4, 0.3 unit of T4 DNA ligase (BRL). Oligonucleotides were separated by denaturing polyacrylamide gel electrophoresis and detected by autoradiography.

the AMP group was rapidly transferred to the oligonucleotide, reaching a maximum between 0 and 45 s. On further incubation the level of the DNA–adenylate intermediate decreased to <5% of the maximum level in 10 min (Figure 4A).

Formation of the DNA–adenylate intermediate was optimal between 20 and 50 mM NaCl with 50% inhibition at 10 and 200 mM. Under similar assay conditions Cdc9 protein did not transfer the adenylate group to either 5′-hydroxyl $(dT)_{16}$ hybridized to poly(dA) or to 5′-phosphorylated $(dT)_{16}$ (data not shown). This result demonstrates the requirement for a 5′-phosphoryl group and a double-stranded structure at the site of the nick. The reactions shown in Figure 4A were carried out in 60 mM Mes–NaOH, pH 6.4. In similar experiments carried out at pH 7.8 (conditions which resulted in approximately the same level of enzyme–adenylate formation as that detected in 60 mM Mes–NaOH, pH 6.4.), the kinetics of the appearance and disappearance of the DNA–adenylate intermediate were identical, but the level of intermediate was reduced about 30-fold (data not shown). Therefore, the difference in the level of the DNA–adenylate intermediate reflects the effect of pH on the rates of the subsequent steps in the reaction. The rate of formation of the DNA–adenylate intermediate can be decreased by reducing the reaction temperature. At 0 °C in 60 mM Mes–NaOH, pH 6.4, the DNA–adenylate intermediate slowly accumulated over a 60-min time period (Figure 4B).

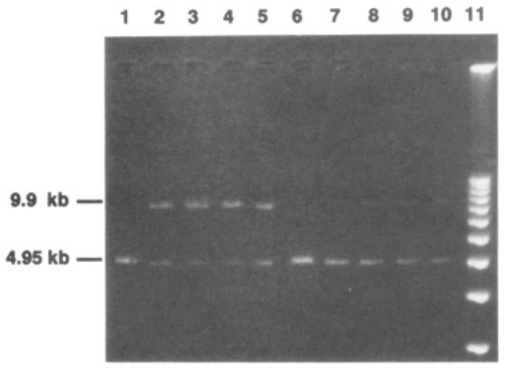

FIGURE 6: Joining of DNA molecules with either short complementary extensions or blunt ends by Cdc9 DNA ligase. Cdc9 DNA ligase (fraction VII, 1.2 μg) was incubated in the assay buffer described in Experimental Procedures at 20 °C with 150 ng of *Pst*I-linearized plasmid molecules for the following times: lane 1, 0 min; lane 2, 30 min; lane 3, 60 min; lane 4, 90 min; lane 5, 120 min; and with 150 ng of *Sma*I-linearized plasmid molecules for the following times: lane 6, 0 min; lane 7, 30 min; lane 8, 60 min; lane 9, 90 min; lane 10, 120 min. Reactions were terminated by the addition of 10 mM EDTA. The molecular mass standard in lane 11 was *Hin*dIII-digested λ DNA (BRL). After electrophoresis through a 0.8% agarose gel, DNA was detected by staining with ethidium bromide.

Interaction of Cdc9 Protein with ATPαS. We examined the ability of Cdc9 protein to interact with the ATP analog ATPαS. Cdc9 DNA ligase (fraction VII) was incubated with [α-^{32}P]ATP or [α-^{35}S]ATP. After separation by SDS–PAGE, adenylated polypeptides were transferred to nitrocellulose. Bands corresponding to Cdc9 protein were detected by staining with amido black and excised, and radioactivity was measured. Approximately 3 times more enzyme–adenylate was formed with [α-^{35}S]ATP than with [α-^{32}P]ATP. Since ATPαS was unable to substitute for ATP in the overall ligation reaction (data not shown), we investigated the transfer of the adenosine 5′-thiomonophosphate group from Cdc9 protein to nicked polynucleotide substrate. In comparison with the transfer of AMP, this reaction was markedly less efficient. There was a slow accumulation of the DNA–[^{35}S]adenylate over a 90-min incubation at 0 °C (Figure 4C), whereas maximum formation of normal DNA–adenylate was detected between 0 and 45 s (Figure 4A), with the intermediate being rapidly utilized in the subsequent formation of phosphodiester bonds. A similar accumulation of the DNA–[^{35}S]adenylate resulted at 20 °C, pH 7.8, over a 40-min time period (data not shown). The stability of the DNA–thioadenylate suggests that this intermediate is less reactive in the final step of the ligation reaction, phosphodiester bond formation.

Ligation of Different Homopolymer Substrates. The ability of Cdc9 protein to catalyze the ligation of various homopolymer substrates was examined. Ligation products were detected by denaturing polyacrylamide gel electrophoresis and by the formation of phosphatase-resistant diesters as described in Experimental Procedures. Both intact Cdc9 DNA ligase and the 70-kDa proteolytic fragment catalyzed the joining of oligo-(dT) molecules hybridized to poly(dA) (Figure 5A, lanes 2 and 3) and oligo(rA) molecules hybridized to poly(dT) (Figure

Table II: Oligonucleotide Substrates Containing a Defined Nick[a]

```
5′          OLIGO #1          3′5′          OLIGO #2       3′
C-A-A-G-C-T-T-G-C-A-T-G-C-C-T-G-C-A G-G-T-C-G-A-C-T-C-T-A-G-A-G-G-A
G-T-T-C-G-A-A-C-G-T-A-C-G-G-A-C-G-T-C-C-A-G-C-T-G-A-G-A-T-C-T-C-C-T
3′                              OLIGO #3                        5′

5′          OLIGO #4          3′5′          OLIGO #5          3′
A-C-G-C-C-A-A-G-C-T-T-G-C-A-T-G-C-C T-G-C-A-G-G-T-C-G-A-C-T-C-T-A-G-A-G-G-A
T-G-C-G-G-T-T-C-G-A-A-C-G-T-A-C-G-G-A-C-G-T-C-C-A-G-C-T-G-A-G-A-T-C-T-C-C-T
3′                              OLIGO #6                        5′
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[a] The polynucleotide substrates shown and derivatives with altered termini at the single-strand nick were end-labeled and hybridized as described in Experimental Procedures.

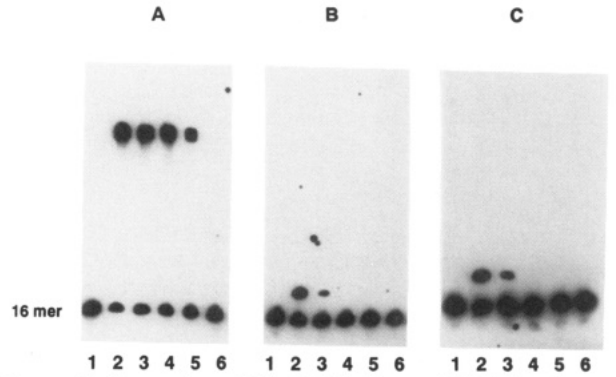

FIGURE 7: Interaction of Cdc9 DNA ligase with DNA substrates containing a single nick or gap. Ligation assays, which were carried out as described in Experimental Procedures, contained the following: lane 1, no enzyme; lane 2, 4.5 μg of Cdc9 DNA ligase (fraction VII); lane 3, 0.45 μg of Cdc9 DNA ligase (fraction VII); lane 4, 45 ng of Cdc9 DNA ligase (fraction VII); lane 5, 4.5 ng of Cdc9 DNA ligase (fraction VII); and lane 6, 0.45 ng of Cdc9 DNA ligase (fraction VII). The polynucleotide substrates (Table II) were as follows: (A) Oligo #1 and ^{32}P-labeled oligo #2 hybridized to oligo #3. (B) Oligo #1 with a 1-base 3′ deletion and ^{32}P-labeled oligo #2 hybridized to oligo #3. (C) Oligo #1 with a 3′-dideoxy adenine residue and ^{32}P-labeled oligo #2 hybridized to oligo #3. Incubations were at 20 °C for 30 min. Oligonucleotides were separated by denaturing polyacrylamide gel electrophoresis and detected by autoradiography.

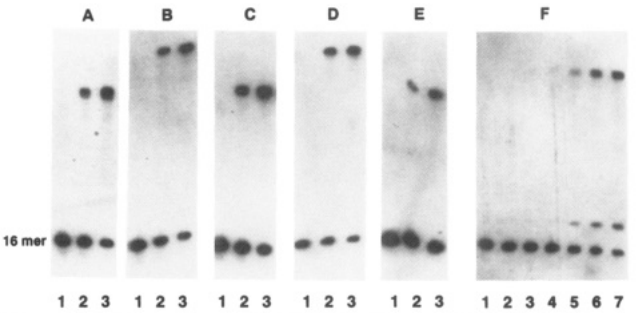

FIGURE 8: Interaction of Cdc9 DNA ligase with DNA substrates containing a single nick with 3′ and/or 5′ mismatches opposite pyrimidines. Ligation assays with the following DNA substrates (Table II) were carried out as described in Experimental Procedures. (A) Oligo #1 and ^{32}P-labeled oligo #2 hybridized to oligo #3. (B) Oligo #1 with a 3′ guanine residue and ^{32}P-labeled oligo #2 hybridized to oligo #3. (C) Oligo #1 with a 3′ cytosine residue and ^{32}P-labeled oligo #2 hybridized to oligo #3. (D) Oligo #1 and ^{32}P-labeled oligo #2 with a 5′ thymine residue hybridized to oligo #3 (Table II). (E) Oligo #1 and ^{32}P-labeled oligo #2 with a 5′ adenine residue hybridized to oligo #3. (F) Oligo #1 with a 3′ cytosine residue and oligo #2 with a 5′ thymine residue hybridized to oligo #3. Reactions were incubated at 20 °C for 30 min with the following: lane 1, no enzyme; lane 2, 3 ng of Cdc9 DNA ligase (fraction VII); lane 3, 9 ng of Cdc9 DNA ligase (fraction VII); lane 4, 30 ng of Cdc9 DNA ligase (fraction VII); lane 5, 90 ng of Cdc9 DNA ligase (fraction VII); lane 6, 300 ng of Cdc9 DNA ligase (fraction VII); and lane 7, 900 ng of Cdc9 DNA ligase (fraction VII). Oligonucleotides were separated by denaturing polyacrylamide gel electrophoresis and detected by autoradiography. Since the samples were run on different gels, the autoradiographs are aligned with respect to ^{32}P-labeled oligo #2 (16 mer). Variations in the running time of the gels cause the differences in position of the upper band corresponding to a 34 mer.

5B, lanes 2 and 3). However, unlike bacteriophage T4 DNA ligase, the yeast enzyme did not catalyze the ligation of oligo(dT) molecules hybridized to poly(rA) (Figure 5A, lanes 6 and 7). Cdc9 DNA ligase was ~2-fold more active on oligo(rA)·poly(dT) than on oligo(dT)·poly(dA) in assays which measured ligation by the formation of acid-precipitable ^{32}P-labeled phosphatase-resistant diesters, whereas it was less then 1/10000 as active on an oligo(dT)·poly(rA) substrate (data not shown). These results are consistent with the observation that the Cdc9–adenylate intermediate transferred the AMP group to oligo(dT)·poly(dA), but not to oligo(dT)·poly(rA) (Figure 2B, lane 4).

In contrast to the adenylylation reactions, the complete ligation reaction was extremely sensitive to the concentration of monovalent ions. Maximum activity was measured at 1 mM NaCl, with 50% inhibition at 30 mM NaCl. This suggests that phosphodiester bond formation on the homopolymer substrate is rate limiting at physiological salt concentrations. The ligation reaction had a pH optimum of 7.6 and was more efficient in Hepes–NaOH compared with Tris-HCl. Since the ligation reaction was 5–6 times more efficient in Tris-HCl, pH 7.8, than in Mes–NaOH, pH 6.4., it can be concluded that the latter stages of the reaction are more readily inhibited by acidic conditions.

In addition to homopolymer substrates, the ability of Cdc9 to join sticky and blunt-ended DNA restriction fragments was examined. The enzyme ligated DNA fragments with short complementary extensions (Figure 6, lanes 2–5). In addition, Cdc9 DNA ligase joined blunt-ended DNA fragments, although this reaction was less efficient than the reaction with sticky-ended restriction fragments (Figure 6, lanes 7–10).

Interaction of Cdc9 DNA Ligase with Polynucleotide Substrates. The differential activity of DNA ligases with various homopolymer substrates has provided biochemical criteria for distinguishing between different mammalian DNA ligases. However, these substrates are not identical to those utilized in vivo. The reactivity of Cdc9 DNA ligase with a polynucleotide substrate containing a single, stable nick with 3′-hydroxyl and 5′-phosphoryl termini (Table II) is shown in Figure 7A. In comparison with this substrate, the enzyme was unable to join 3′-hydroxyl and 5′-phosphoryl termini separated by a one-nucleotide gap, although the slower migrating DNA–adenylate intermediate was formed at higher enzyme concentrations (Figure 7B). Formation of the DNA–adenylate intermediate was greater than 10-fold reduced when the substrate contained a two-nucleotide gap (data not shown). These results indicate that the enzyme recognizes both the 3′ and 5′ termini prior to transfer of the adenylate group. The

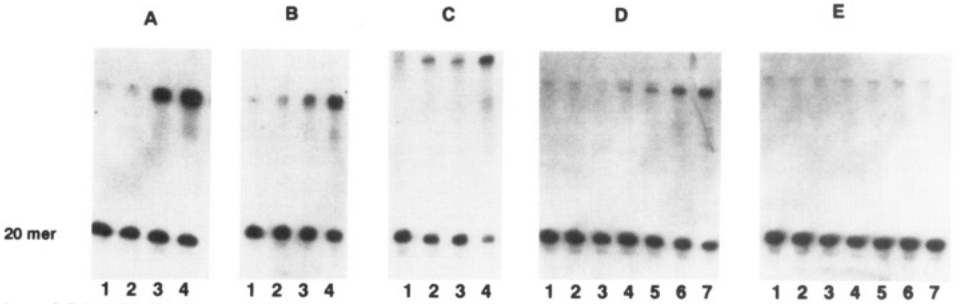

FIGURE 9: Interaction of Cdc9 DNA ligase with DNA substrates containing a single nick with 3′ or 5′ mismatches opposite purines. Ligation assays with the following DNA substrates (Table II) were carried out as described in Experimental Procedures. (A) Oligo #4 and ^{32}P-labeled oligo #5 were hybridized with oligo #6. (B) Oligo #4 and ^{32}P-labeled oligo #5 with a 5′ guanine residue were hybridized with oligo #6. (C) Oligo #4 and ^{32}P-labeled oligo #5 with a 5′ cytosine residue were hybridized with oligo #6. (D) Oligo #4 with a 3′ thymine residue and ^{32}P-labeled oligo #5 were hybridized with oligo #6. (E) Oligo #4 with a 3′ adenine residue and ^{32}P-labeled oligo #5 were hybridized with oligo #6. Reactions were incubated at 20 °C for 30 min with the following: lane 1, no enzyme; lane 2, 3 ng of Cdc9 DNA ligase (fraction VII); lane 3, 9 ng of Cdc9 DNA ligase (fraction VII); lane 4, 30 ng of Cdc9 DNA ligase (fraction VII); lane 5, 90 ng of Cdc9 DNA ligase (fraction VII); lane 6, 300 ng of Cdc9 DNA ligase (fraction VII); and lane 7, 900 ng of Cdc9 DNA ligase (fraction VII). Oligonucleotides were separated by denaturing polyacrylamide gel electrophoresis and detected by autoradiography. Since the samples were run on different gels, the autoradiographs are aligned with respect to ^{32}P-labeled oligo #5 (20 mer). Variations in the running time of the gels cause the differences in position of the upper band corresponding to a 38 mer.

detection of DNA–adenylate intermediate in the substrate with a single nucleotide gap is presumably a consequence of the physical separation between the 3′-hydroxyl group, which acts as a nucleophile in subsequent phosphodiester bond formation, and the 5′ DNA–adenylate moiety. The essential role of the 3′-hydroxyl in phosphodiester bond formation was addressed directly by constructing a substrate with a dideoxy nucleotide at the 3′ terminus of the nick. Cdc9 DNA ligase was able to transfer the adenylate group to the 5′ phosphate terminus. However, once again phosphodiester bond formation was inhibited (Figure 7C).

The ability of Cdc9 DNA ligase to discriminate between correct and mispaired bases at the 3′ and 5′ termini of a nick was also examined. Mismatches opposite pyrimidines in the template strand and located at either terminus did not significantly inhibit the ligation reaction (Figure 8A–E). A similar effect was observed with 5′ mismatched bases opposite purines in the template strand (Figure 9A–C). The efficiency of ligation of substrates with mismatched bases opposite pyrimidines and located at both the 5′ and 3′ termini was reduced by greater than 100-fold (Figure 8F). Mismatched bases opposite purines in the template strand and located at the 3′ terminus had a profound effect on the ability of Cdc9 DNA ligase I to catalyze phosphodiester bond formation. A thymine–guanine mismatch reduced the extent of ligation by 50-fold (Figure 9D), and an adenine–guanine mismatch completely abolished ligation (Figure 9E). The absence of accumulated DNA–adenylate intermediate suggests that these 3′ mismatches inhibited ligation by interfering with recognition of the 5′ terminus.

DISCUSSION

The ability of human DNA ligase I cDNA to complement defective DNA replication (Barnes et al., 1990), DNA repair,[1] and mitotic recombination[1] in a *cdc9* mutant of *S. cerevisiae* indicates that these enzymes fulfill similar, if not identical, roles in eukaryotic cells. In this study we have purified the DNA ligase activity encoded by the *CDC9* gene (Barker et al., 1973) to >95% homogeneity. *S. cerevisiae* Cdc DNA ligase is an 87-kDa polypeptide as determined by SDS–PAGE. This estimate is in good agreement with the M_r of 84 406 calculated from the translated nucleotide sequence of the gene (Barker et al., 1983).

The physical and structural properties of Cdc9 DNA ligase closely resemble those of other ATP-dependent DNA ligases. In all cases the native enzyme is an asymmetric monomer, a property that results in anomalous estimates of molecular mass from gel filtration and sedimentation experiments (Kornberg & Baker, 1989; Tomkinson et al., 1990, 1991a; Lindahl & Barnes, 1992). Additionally, the intact enzyme is readily cleaved by either endogenous or reagent proteolytic enzymes, into a C-terminal fragment that retains the catalytic properties of the native enzyme (Rabin et al., 1986; Tomkinson et al., 1990). Both mammalian DNA ligase I and Cdc9 protein are cleaved at similar sites if the proteins are aligned from their C-termini (Barker et al., 1983; Barnes et al., 1990; Tomkinson et al., 1990). These observations are consistent with the hypothesis that ATP-dependent DNA ligases contain a conserved C-terminal catalytic domain with N-terminal extensions of varying length (Barnes et al., 1990; Tomkinson et al., 1990). The role of the N-terminal region of these enzymes is not known. It has been speculated that it may be involved in protein–protein interactions and/or posttranslational modification (Tomkinson et al., 1990).

S. cerevisiae Cdc9 DNA ligase acts by the same reaction mechanism utilized by all known DNA ligases. In the initial reaction the viral and eukaryotic enzymes interact with ATP, whereas bacterial enzymes interact with NAD, to form an enzyme–adenylate intermediate (Gumport & Lehman, 1971; Soderhall & Lindahl, 1973; Rabin & Chase, 1987; Tomkinson et al., 1991a). The AMP group is linked via a phosphoramidate bond to a lysine residue (Gumport & Lehman, 1971; Soderhall & Lindahl, 1973). This active site residue has been identified in mammalian DNA ligase I, allowing the putative assignment of the active site lysine residue in *S. cerevisiae* Cdc9 protein by sequence homology (Tomkinson et al., 1991b).

In contrast to this first step of the ligation reaction, relatively little is known about the mechanisms of the subsequent steps that involve the interaction of eukaryotic DNA ligase with a polynucleotide substrate. We have examined the effects of alterations of reaction conditions and cofactors on the transfer of the adenyl group from the enzyme to the 5′-phosphorylated terminus of the polynucleotide substrate. In agreement with earlier studies on the formation of DNA–adenylate (Olivera et al., 1968; Soderhall, 1975) we found that this intermediate was more readily detected in reactions at low pH and low temperature. Bacteriophage T4 DNA ligase, DNA ligase I from mammalian cells, and *S. cerevisiae* Cdc9 DNA ligase form enzyme–adenylate intermediates with the ATP analog ATPαS, but this cofactor cannot substitute for ATP in the

overall DNA ligation reaction (Elder & Rossignol, 1990; Montecucco et al., 1990). The replacement of the oxygen with a sulfur atom at the α-phosphate group inhibits both the transfer of the adenosine 5′-thiomonophosphate group from the enzyme to the 5′-phosphoryl group of the polynucleotide substrate and the subsequent reaction utilizing the DNA–thioadenylate intermediate to form a phosphodiester bond.

Cdc9 DNA ligase and mammalian DNA ligase I exhibit the same polynucleotide substrate specificity. Both enzymes join oligo(dT) molecules hybridized to poly(dA) and oligo(rA) hybridized to poly(dT), but not oligo(dT) hybridized to poly(rA) (Arrand et al., 1986; Tomkinson et al., 1991a). Additionally, both enzymes join DNA molecules with blunt ends and/or short complementary extensions (Arrand et al., 1986). Despite the similarities in physical and catalytic properties and the conservation of amino acid sequence between mammalian DNA ligase I and Cdc9 protein, polyclonal antibodies raised against bovine DNA ligase I (Tomkinson et al., 1990) do not cross-react with Cdc9 protein.

Since Cdc9 DNA ligase has very similar physical and biochemical properties to DNA ligase I of *Drosophila* (Rabin et al., 1986; Rabin & Chase, 1987) and mammalian cells (Tomkinson et al., 1990, 1991a), the product of the yeast *CDC9* gene can be designated as *S. cerevisiae* DNA ligase I. *Drosophila* (Takahashi & Tomizawa, 1990) and mammalian cells (Tomkinson et al., 1991a; Lindahl & Barnes, 1992) contain additional species of DNA ligase, but the cellular roles of these enzymes have not been determined. *S. cerevisiae* is an attractive model eukaryotic system for studying the cellular roles of multiple DNA ligases because of the potential for identifying genes which encode such enzymes and for isolating mutants. Indeed, during the course of the purification of yeast DNA ligase I we have identified another DNA ligase activity that acts on an oligo(dT)·poly(rA) substrate (data not shown). This activity, which may be functionally homologous to mammalian DNA ligase II or III, is under further study.

There is genetic evidence that certain DNA ligases may play a specific role in error-prone DNA repair. T4 DNA ligase mutants are hypomutable by ultraviolet radiation and by nitrous oxide (Yarosh, 1978). A human cell line designated 46 BR, which has mutations in the DNA ligase I gene, is also hypomutable by ultraviolet and ionizing radiation (Henderson et al., 1985). In contrast, the deficiency of DNA ligase in a *Escherichia coli* DNA ligase mutant causes the induction of error-prone DNA repair (Morse & Pauling, 1975). A possible explanation for these observations is that DNA ligase causes mutations by sealing nicks which have mismatches at the termini. *S. cerevisiae* DNA ligase I was not significantly inhibited by 3′ or 5′ mismatches opposite pyrimidines or by 5′ mismatches opposite purines, at the site of nicks. The insensitivity to 5′ mismatches is consistent with the observation that *Drosophila* DNA ligase I can join oligonucleotides with a single 5′ mismatch when hybridized to a homopolymer template (Rabin & Chase, 1987).

In contrast, the joining of DNA molecules with 3′ mismatches opposite a purine at the site of a nick was inhibited, and the greatest effect was observed with purine–purine mismatches. In these cases the mismatches interfered with the interaction of the enzyme with the 5′ terminus, suggesting a steric effect rather than the ability of the enzyme to specifically recognize the absence of base pairing at the 3′ terminus. DNA molecules containing nicks with mismatches at the 3′ terminus may be generated by misincorporation either during lagging strand DNA synthesis or after gap filling following excision repair. Mismatches produced during DNA replication are expected to be substrates for the mismatch DNA repair system. However, those generated at the 3′ terminus after gap-filling may contribute to the class of nontargeted mutations produced by error-prone DNA repair. The ability of eukaryotic DNA ligase I (and under certain conditions T4 DNA ligase) to seal nicks with mismatched termini correlates with the introduction of mutations by error-prone DNA repair, suggesting that these enzymes play a direct role in mutagenesis. The joining of DNA molecules with mismatched termini may be involved in other physiological processes, such as the generation of diversity at the coding joint during V(D)J recombination (Lewis & Gellert, 1989). In support of this idea, the single known individual with a mutated DNA ligase I gene in the cell line 46 BR was severely immunodeficient (Barnes et al., 1992).

ACKNOWLEDGMENT

We thank Dr. Clive Slaughter and Carolyn Moomaw of the Howard Hughes Medical Institute, The University of Texas Southwestern Medical Center, for the amino acid sequencing and Dr. Stephen Johnston, Division of Molecular Cardiology, The University of Texas Southwestern Medical Center, for oligonucleotide synthesis. We also thank Doug Johnson for technical assistance, Karla Henning for assistance with yeast transformation, and our laboratory colleagues for their critical review of the manuscript.

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