19
DNA Ligases

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DNA ligases are Mg^{++}-dependent enzymes that catalyze the formation of phosphodiester bonds at single-strand breaks in double-stranded DNA (for review and main references up to 1992, see Engler and Richardson 1982; Lindahl and Barnes 1992). The first step in the reaction is the formation of a covalent enzyme/adenylate intermediate. DNA ligases from eukaryotes, archaea, and viruses employ ATP as cofactor, whereas eubacterial DNA ligases use NAD to generate the adenylyl group. The ATP is cleaved to AMP and pyrophosphate with the adenylyl residue linked by a phosphoramidate bond to the ε-amino group of a specific lysine residue at the active site of the protein. The reaction is readily reversed in vitro by addition of pyrophosphate. Since DNA ligases contain an unusually reactive lysine residue in their active site, a Schiff base can be formed with pyridoxal phosphate. In consequence, the activities of DNA ligases, e.g., mammalian DNA ligases I and II, are inhibited in vitro by pyridoxal phosphate. ATP-dependent DNA ligases can employ certain cofactor analogs such as dATP, but the anomalous enzyme/nucleotide complexes formed by some ligases appear to function poorly in subsequent steps of the DNA-joining reaction. The activated AMP residue of the DNA ligase/adenylate intermediate is transferred to the 5′-phosphate terminus of a single-strand break in double-stranded DNA to generate a covalent DNA-AMP complex with a 5′–5′ phosphoanhydride bond. In the final step of DNA ligation, unadenylylated DNA ligase is required for the generation of a phosphodiester bond and catalyzes displacement of the AMP residue through attack by the adjacent 3′-hydroxyl group on the adenylylated site (Fig. 1).

The active site of mammalian DNA ligase I has been identified by isolation and amino acid sequencing of the tryptic peptide containing a lysine-[^{3}H]AMP moiety and localized by comparison with the complete peptide sequence deduced from the cDNA. The amino acid sequence of the peptide containing the active-site lysine residue has also been

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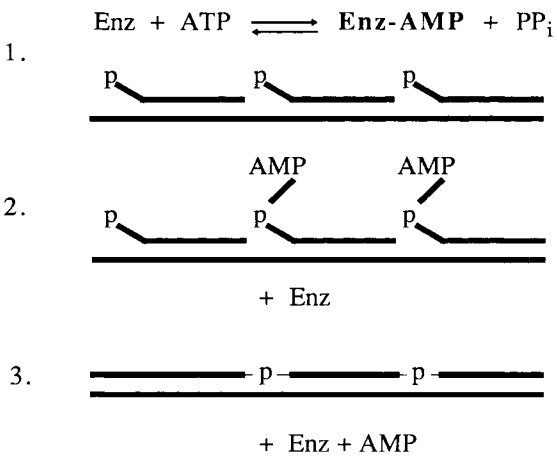

Figure 1 The reaction catalyzed by DNA ligases. The enzyme/adenylate intermediate is highlighted in bold.

determined for mammalian DNA ligase II (Wang et al. 1994). Comparisons with nucleic acid sequences encoding DNA ligases of other species have identified a ubiquitous K*X*DG active-site motif. This motif has also been found at the active site of the vaccinia virus mRNA-capping enzyme that catalyzes transfer of GMP from GTP to the 5′ terminus of RNA and forms an enzyme/guanylate reaction intermediate with a lysine/GMP phosphoramidate bond. The observed sequence conservation indicates a common mechanism of covalent catalysis in these nucleotidyl transfer reactions (Cong and Shuman 1993).

Some DNA ligases are able to catalyze blunt-end joining in addition to ligating nicks in double-stranded DNA. Blunt-end joining proceeds less efficiently than ligation of nicks or cohesive ends. Mammalian DNA ligase I effectively seals single-strand breaks in DNA and joins restriction enzyme DNA fragments with staggered ends. The enzyme is also able to catalyze blunt-end joining of DNA. As for other DNA ligases, the latter reaction is stimulated greatly by the macromolecular crowding conditions inflicted by the presence of polyethylene glycol in reaction mixtures. DNA ligase I is more effective at blunt-end joining than mammalian DNA ligases II and III but is less efficient in this regard than bacteriophage T4 DNA ligase.

ASSAYS

Formation of an Enzyme/Adenylate Intermediate

This assay depends on the enzyme's ability to covalently bind AMP. If the DNA ligase is incubated with [α-^{32}P]ATP, the enzyme/AMP inter-

mediate is radiolabeled. These complexes are stable in the absence of pyrophosphate or DNA substrate. The enzyme/adenylate can then be separated by SDS-PAGE and visualized using autoradiography. This method is useful in determining which DNA ligases are present in partially purified protein fractions, as the respective enzyme/AMP intermediates migrate differently according to their molecular masses. Figure 2 illustrates this point by showing mammalian DNA ligases I, II, and III, (lanes a, b, and c, respectively), labeled at their active sites with [α-^{32}P]ATP. The proteins are then fractionated by SDS-gel electrophoresis to reveal apparent molecular masses of 125 kD, 69 kD, and 100 kD, respectively. Due to anomalously slow migration of DNA ligase I, the first number is a slight overestimate in comparison with data deduced from the cDNA open reading frame.

Specific Dissociation of an Enzyme/Adenylate Intermediate by Nicked DNA

The adenylate group can be specifically removed from the complex either by transfer to a 5′-phosphate acceptor of a polynucleotide substrate or by incubation with pyrophosphate, reversing the initial reaction to

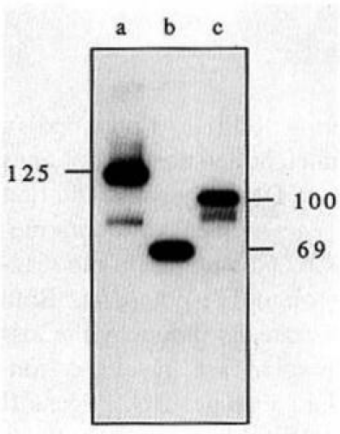

Figure 2 Adenylylation of DNA ligases I, II, and III. DNA ligase I (*a*), DNA ligase II (*b*), and DNA ligase III (*c*) were incubated for 15 min at room temperature in a standard reaction mixture (10 µl), containing 60 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, 50 µg/ml bovine serum albumin, and 0.5 µCi [α-^{32}P]ATP (3000 Ci/mmole, Amersham). 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. Gels were fixed for 10 min in 10% acetic acid, and dried. Adenylylated proteins were detected by autoradiography.

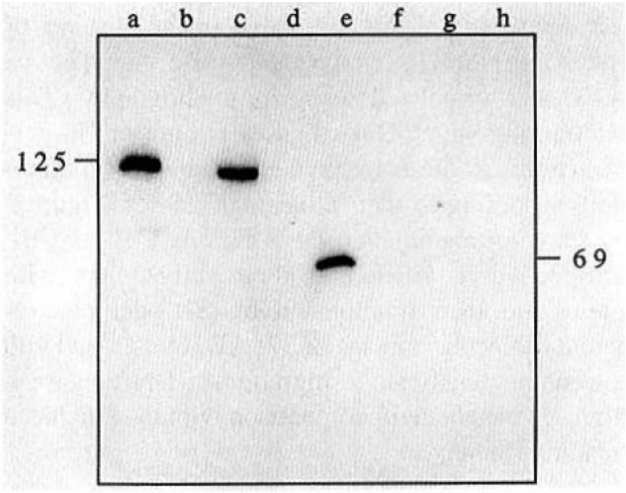

Figure 3 Reactivity of the adenylylated forms of DNA ligases I and II. DNA ligase I (*a–d*) and DNA ligase II (*e–h*) adenylate intermediates were formed, as described in Fig. 2, in a final volume of 40 μl. They were then further incubated with unlabeled polynucleotide substrate or sodium pyrophosphate. Aliquots (10 μl) were incubated with no addition (*a, e*); 0.8 μg unlabeled oligo(dT)•poly(dA) (*b, f*); 0.8 μg unlabeled oligo(dT)•poly(rA) (*c, g*); or 10 nmoles of sodium pyrophosphate (*d, h*) for 1 hr at 37°C. The reactions were stopped by the addition of SDS sample buffer, and adenylylated polypeptides were detected as described in Fig. 2.

regenerate ATP. Figure 3 illustrates the differing abilities of mammalian DNA ligases I and II to react with two polynucleotide substrates. Lanes a–d refer to DNA ligase I, and lanes e–h refer to DNA ligase II. The first lane in each set of four shows the respective enzyme/adenylate intermediate prior to addition of any substrate. Lanes b and f represent the reaction of the radiolabeled intermediate with oligo(dT)•poly(dA). Both DNA ligases I and II are able to ligate this substrate, as shown by the loss of radiolabeled enzyme/AMP intermediate in each case, resulting from transfer of the radiolabeled AMP group to the substrate. DNA ligase II (lane g), but not DNA ligase I (lane c), is also able to react with an oligo(dT)•poly(rA) substrate. Lanes d and h show reversal of the first step in the reaction by the addition of pyrophosphate.

Formation of Joined Oligonucleotides

In this case, the same substrates are used as in the previous assay, except that the oligonucleotide is radioactively labeled at its 5′ end with ^{32}P.

The enzyme is incubated, in the presence of Mg^{++} and ATP, with the substrate, e.g., 5′-^{32}P-labeled oligo(dT) hybridized to either poly(dA) or poly(rA). Multimers of the oligonucleotides are subsequently detected by autoradiography after electrophoresis through a denaturing polyacrylamide gel.

Ligation of Nicked Circular DNA to Covalently Closed Circular DNA

This system provides a striking and convincing demonstration of DNA ligase activity. However, in contrast to the assays described above, it does not provide additional information on either the size of the DNA ligase or its substrate specificity with regard to joining DNA-RNA hybrids. To generate the appropriate substrate, covalently closed circular DNA is treated with a very low concentration of pancreatic DNase I, or restriction endonuclease *Eco*RI in the presence of ethidium bromide. The nicked DNA is purified free from remaining covalently closed circular DNA and employed as substrate. The reaction is stopped by addition of EDTA and proteinase K. Rejoining of the DNA strand break by a DNA ligase may be detected by agarose gel electrophoresis in the presence of ethidium bromide.

Blunt-end Joining

The ability of a DNA ligase to join blunt ends can be assayed by the joining of DNA restriction fragments generated by restriction endonucleases such as *Hpa*I that give DNA fragments with fully base-paired ends. Inclusion of 10–20% polyethylene glycol 6000 in standard reaction mixtures may promote the reaction.

Assay Pitfalls

A number of factors can cause problems with the various assays employed to detect DNA ligase activity. Small amounts of contaminating DNase activity in crude cell extracts, or partly purified DNA ligase fractions, may interfere seriously with attempts to detect joining of DNA or oligonucleotides. The assay employing nicked circular DNA as a substrate is particularly sensitive to traces of endonuclease activity. Problems can also be encountered in methods that measure the level of DNA ligation. The presence of exonuclease activities prevents accurate

quantification due to the conversion of nicks to small gaps, which are no longer substrates for DNA ligases. In particular, double-stranded DNA-RNA substrates such as oligo(dT)•poly(rA) are uniquely susceptible to RNase H degradation; RNase H activities are often abundant in crude enzyme fractions. Thus, contamination of a partly purified DNA ligase with RNase H may give the erroneous impression that the DNA ligase can join single-strand breaks in a DNA•DNA, but not a DNA•RNA, double helix.

The formation of a covalent enzyme/adenylate complex in the presence of [α-^{32}P]ATP is a fairly robust and very useful assay, but high ATPase concentrations interfere. Moreover, this is a less sensitive and quantitative method than the DNA ligation assay, since formation of a stoichiometric complex is measured rather than catalytic events. In addition, proteins other than DNA ligases in crude enzyme fractions may become adenylated. In practice, this is rarely a serious problem, but it is imperative to demonstrate that the complex is a DNA ligase/AMP reaction intermediate. This can be shown by establishing that the radioactive moiety is specifically released from the protein by addition of nicked DNA or oligo(dT)•poly(dA) to reaction mixtures.

DNA LIGASE I

An enzyme of this type has been found in all eukaryotic cells investigated. The main function of human DNA ligase I is probably the joining of Okazaki fragments during lagging-strand DNA replication (Waga et al. 1994). The enzyme is also involved in DNA excision repair (Prigent et al. 1994). Similarly to DNA polymerase-α, DNA ligase I is induced 10- to 15-fold in S phase in mammalian cells. Thus, it is present at much higher concentrations in proliferating tissues. A less impressive 3-fold induction has been observed 24–48 hours after cellular exposure to ultraviolet radiation (Montecucco et al. 1992). Immunofluorescence studies with antibodies against DNA ligase I have shown that the enzyme is present in cell nuclei, exhibiting the granular staining also seen for several other replication factors. This may reflect the occurrence of DNA ligase I in "replication factories." A 21S DNA replication complex purified from HeLa cells contained DNA ligase I together with DNA polymerase-α:primase, proliferating cell nuclear antigen, RNase H, and a small number of other proteins (Congjun et al. 1994).

The cloned cDNA for human DNA ligase I encodes a 102-kD protein of 919 amino acid residues. The amino-terminal region of the protein is not required for DNA ligation activity in vitro. This amino-terminal part

of the enzyme is hydrophilic and contains several stretches of either negatively or positively charged amino acid residues. DNA ligase I is a phosphoprotein, and most or all of the phosphate residues are localized to the amino-terminal region. Furthermore, the amino-terminal part is highly susceptible to proteolysis, so a 78-kD active fragment of mammalian DNA ligase I, comprising the catalytic domain of the enzyme, is often generated as a preparation artifact due to endogenous degradation during enzyme purification.

Large amounts of active human DNA ligase I can be obtained by expression of the cDNA in a baculovirus expression system (Wang et al. 1994). The cDNA has also been expressed in *Escherichia coli*, but it is controversial whether such an unphosphorylated form of the enzyme retains all the key features of the native enzyme purified from mammalian cells. However, the subcloned catalytic domain of human DNA ligase I is sufficient to complement a conditional-lethal DNA ligase mutant of *E. coli*. The properties of the amino-terminal region of DNA ligase I indicate that it may serve in specific binding of the enzyme to other DNA replication and repair proteins. It is not required for interactions with either nicked DNA or the ATP cofactor. The active-site lysine residue is centrally located within the catalytic domain of the enzyme. cDNA deletion experiments have shown that although 250 amino acid residues can be removed from the amino-terminal region of the protein without loss of enzyme activity, only 16 residues can be deleted at the carboxyl terminus before an essential, highly conserved motif is encountered.

The human gene encoding DNA ligase I is located at chromosome 19q13.2-13.3. The gene covers 53 kb and contains 28 exons (Noguiez et al. 1992). Unusually for an enzyme involved in DNA replication, an individual has been identified with an inherited defect in DNA ligase I (Barnes et al. 1992). The clinical symptoms were severe immunodeficiency, stunted growth, and sun sensitivity. The individual died at a young age from an abdominal lymphoma with an associated acute infection (Webster et al. 1992). A fibroblast cell strain, 46BR, derived from the individual shows deleterious mutations in both alleles of the DNA ligase I gene. One has an inactivating mutation in the active-site region, whereas the other has a mutation in a conserved part of the carboxy-terminal region that allows a small amount of residual DNA ligase activity.

The leakiness of the latter point mutation probably accounts for the fact that a mutation in a DNA replication enzyme is compatible with cellular viability. An SV40-transformed subline, 46BR•1G1, is available

that contains only the latter, leaky mutation expressed in hemizygous or homozygous form. 46BR cells show inefficient joining of Okazaki fragments and anomalous gap filling during excision repair (Prigent et al. 1994). However, V(D)J joining seems to proceed normally in 46BR cells (Hsieh et al. 1993). Since the 46BR patient was sun sensitive, and the late steps of lagging-strand DNA replication and nucleotide excision repair share many factors (Aboussekhra et al. 1995), it seems likely that DNA ligase I is responsible for the final DNA-joining step in nucleotide excision repair.

The *Saccharomyces cerevisiae CDC9* and *Schizosaccharomyces pombe cdc17*$^+$ gene products appear to be the yeast counterparts of mammalian DNA ligase I. Thus, the purified *S. cerevisiae* and *S. pombe* enzymes have catalytic properties practically identical to those of the mammalian enzyme, whereas they differ from mammalian DNA ligases II and III (Tomkinson et al. 1992; P. Schär, unpubl.). The 86–87-kD yeast enzymes also have an amino-terminal region, shorter than that of the mammalian enzyme, which is very susceptible to proteolysis and nonessential for DNA ligation activity in vitro. The overall sequence homology between human DNA ligase I and the yeast enzymes is about 40%; however, no similarity is detected between the sequences of the amino-terminal regions of these three enzymes, whereas high homology (70%) is observed in the region surrounding the active-site lysine residue.

Conditional-lethal yeast mutants with a temperature-sensitive DNA ligase accumulate non-joined DNA fragments during abortive replication at the restrictive temperature and show defects in DNA repair and recombination at the permissive temperature. Presently, it is unclear whether *S. cerevisiae* and *S. pombe* contain enzymes similar to mammalian DNA ligases II and III. They may not yet have been detected, but it is also possible that their functions are not required in yeast cells or that the *CDC9*- and *cdc17*$^+$-encoded enzymes also perform the corresponding roles of mammalian DNA ligases II and III.

DNA ligase I from *Drosophila melanogaster* and *Xenopus laevis* also has biochemical properties very similar to those of mammalian DNA ligase I. Both these enzymes resemble mammalian DNA ligase I in having a protease-sensitive region not required for ligation activity. The intact *Drosophila* enzyme is an 83- to 86-kD protein, similar in size to the yeast enzyme. In contrast, the *Xenopus* enzyme has a catalytic domain of about the same size as that of the mammalian enzyme but an unusually long, phosphorylated amino-terminal region. Thus, *Xenopus* DNA ligase I migrates as an apparent 180-kD protein on SDS-PAGE (Aoufouchi et al. 1992).

DNA LIGASE II

This approximately 69-kD DNA ligase can be distinguished from DNA ligase I by its ability to join an oligo(dT)·poly(rA) substrate. DNA ligase II is not induced on cell proliferation, and its cellular role is not clear. It is the major DNA ligase activity in certain nonproliferating tissues, e.g., adult liver. The enzyme is more firmly retained in cell nuclei than DNA ligase I and requires buffers of moderate or high salt concentration for efficient extraction. Neutralizing rabbit antibodies against DNA ligase I do not detectably recognize or inhibit DNA ligase II, and, similarly, neutralizing antibodies against DNA ligase II do not inactivate DNA ligase I. The two enzymes generate entirely different fragmentation patterns on proteolysis, and there is no indication of a protease-sensitive amino-terminal region (Roberts et al. 1994). Recently, the amino acid sequences of the active-site peptide and several other peptides of bovine DNA ligase II were determined (Wang et al. 1994). These show only about 30% homology between mammalian DNA ligases I and II, although main active-site features common to all ATP-dependent DNA ligases are present. Like DNA ligase I and most nuclear proteins, DNA ligase II has a blocked amino-terminal amino acid residue. Interestingly, mammalian DNA ligase II is much more similar to the vaccinia virus-encoded DNA ligase than to DNA ligase I (Wang et al. 1994). *D. melanogaster* also has a DNA ligase II, which differs from DNA ligase I in being present in similar amounts during developmental stages with high (early embryos) or low (pupae, adults) DNA replication rates.

DNA LIGASE III

DNA ligase III is a mammalian DNA ligase of 103 kD. A cDNA has been cloned, and the gene for DNA ligase III has been mapped to human chromosome 17q11.2-12 (Wei et al. 1995). The enzyme resembles DNA ligase I in having a protease-sensitive amino-terminal region not required for DNA ligation activity in vitro, but it resembles DNA ligase II in its ability to join an oligo(dT)·poly(rA) substrate. Sequences of DNA ligase III and the smaller DNA ligase II seem identical or closely similar, so both these forms of the enzyme may be derived from the same gene (Roberts et al. 1994; Husain et al. 1995; Wei et al. 1995). Although DNA ligase III is of similar size to DNA ligase I, it elutes well before the asymmetric DNA ligase I on gel filtration of crude enzyme fractions, indicating that it is a dimer or binds a separate nuclear protein in a salt-resistant interaction. A prime candidate for this partner of DNA ligase III is the 70-kD XRCC1 protein, because the two proteins bind each other in

vitro (Caldecott et al. 1994). Moreover, the Chinese hamster cell line EM9, which is defective in XRCC1 protein, also exhibits an anomalously low level of DNA ligase III activity (Caldecott et al. 1994; Ljungquist et al. 1994). The phenotype of XRCC1 involves a high level of sister-chromatid exchange and hypersensitivity to alkylating agents and ionizing radiation. The latter observations suggest a role for DNA ligase III in base excision repair. A mammalian protein complex that performs recombination events in vitro has been reported to contain DNA ligase III but no detectable DNA ligase I (Jessberger et al. 1993).

DNA LIGASE IV

A fourth mammalian DNA ligase has been detected recently by a search of expressed sequence tags with short sequence motifs highly conserved in eukaryotic DNA ligases (Wei et al. 1995). The complete cDNA encodes a 96-kD protein with DNA ligase activity that exhibits partial sequence homology with ligases I–III. The human gene for DNA ligase IV is localized on chromosome 13q33-34. Northern blots indicate that the enzyme is expressed in thymus and testis and at a very low level in several other tissues, but its physiological role is not known. An unusual feature of DNA ligase IV, in comparison with the other mammalian DNA ligases, is an extended carboxy-terminal region of more than 300 amino acids that shows no homology with other proteins in databases. This region may conceivably be involved in protein-protein interactions that could functionally distinguish this enzyme from the other DNA ligases. On SDS-PAGE, DNA ligases III and IV migrate at indistinguishable rates as approximately 100-kD proteins. Thus, an enzyme/AMP complex of this size could be due to either ligase III or IV, making further identification necessary by immunological methods or by exploring the interaction with the XRCC1 protein unique to DNA ligase III. A comparison of the properties of DNA ligases I, II, III, and IV is shown in Table 1.

VIRUS-ENCODED DNA LIGASES

Herpesviruses and smaller DNA and RNA animal viruses do not encode a DNA ligase. However, a distinct virus-encoded enzyme is produced by vaccinia virus and a number of other poxviruses. The 63-kD vaccinia protein only shows weak homology (~30%) with DNA ligase I from human cells, *S. pombe*, or *S. cerevisiae*. Although poxviruses replicate in the host-cell cytoplasm, the vaccinia DNA ligase is nonessential for viral

Table 1 Properties of four mammalian DNA ligases

	I	II	III	IV
Molecular mass estimated				
by cDNA sequence	102 kD	—	103 kD	96 kD
by SDS-PAGE	125 kD	70 kD	100 kD	100 kD
Chromosomal localization	19q13.2–13.3	—	17q11.2–12	13q33–34
Ligation of				
oligo(dT)•poly(dA)	yes	yes	yes	yes
oligo(dT)•poly(rA)	no	yes	yes	yes
oligo(rA)•poly(dT)	yes	no	yes	no
K_m for ATP	1 μM	40 μM	2 μM	—
Function	lagging-strand replication, nucleotide excision repair	—	base excision repair, recombination	—

DNA replication and growth. However, a virus DNA ligase-deficient mutant shows attenuated virulence in vivo and is anomalously sensitive to DNA-damaging agents during infection, implying a role for the enzyme in viral DNA repair. A DNA ligase with a surprisingly different sequence is encoded by African swine fever virus, another large DNA virus replicating in the cytoplasm of infected cells (Hammond et al. 1992). The latter data imply that African swine fever virus is only distantly related to vaccinia virus.

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