DNA Ligase IV from HeLa Cell Nuclei*

Peter Robins and Tomas Lindahl‡

From the Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Hertfordshire EN6 3LD, United Kingdom

A human cDNA encoding a previously unrecognized DNA ligase IV has been identified (Wei, Y.-F., Robins, P., Carter, K., Caldecott, K., Pappin, D. J. C., Yu, G.-L., Wang, R.-P., Shell, B. K., Nash, R. A., Schiar, P., Barnes, D. E., Haseltine, W. A., and Lindahl, T. (1995) Mol. Cell. Biol. 15, 3206–3216). Antibodies have been raised against predicted peptide sequences of DNA ligase IV and used to identify the enzyme during purification from HeLa cell nuclei. The 96-kDa DNA ligase IV and the 103-kDa DNA ligase III co-migrate during SDS-polyacrylamide gel electrophoresis and have similar column fractionation properties, which complicates the distinction between the two enzymes, but they have been separated by Mono S liquid chromatography. During initial size fractionation by gel chromatography in 1 M NaCl, DNA ligase IV elutes in the same position as the DNA ligase III-XRCC1 protein complex, indicating that DNA ligase IV is also bound to another protein or occurs as a dimer. DNA ligase IV has been purified free from other DNA ligases, and its enzymatic properties have been examined. The purified protein effectively joins single-strand breaks in a double-stranded polydeoxynucleotide in an ATP-dependent reaction. The substrate specificity of DNA ligase IV differs from those of the other two cloned human DNA ligases, I and III, with regard to their ability to join the hybrid substrates oligo(dT)poly(rA) and oligo(rA)poly(dT). DNA ligase IV occurs in part as an enzyme-adenylate complex in HeLa cell nuclear extracts.

Previous biochemical studies on fractionated mammalian cell extracts have established the presence of three distinct nuclear DNA ligases (1). The 102-kDa DNA ligase I is not evenly distributed in cell nuclei but is mainly localized in “replication factories” together with DNA polymerase α and other replication factors (2, 3); it is induced in S phase (4) and functions in lagging strand DNA synthesis in a reconstituted SV40 DNA replication system (5). Moreover, a human cell line with a malfunctioning DNA ligase I shows defective joining of Okazaki fragments during lagging strand replication (6), and experiments on homozygous “knockouts” by targeted disruption of the DNA ligase I gene of murine embryonic stem cells (7, 8) indicate this fact.

**EXPERIMENTAL PROCEDURES**

Formation of DNA Ligase-Adenylate—DNA ligase-containing fractions (2 μl) were adenylated in reaction mixtures (10 μl) containing 60 mM Tris-Cl (pH 8.0), 10 mM MgCl₂, 5 mM DTT, 2 μCi of [α-32P]ATP (3000 Ci/mmol; Amersham Corp.) at 20°C for 10 min. Reactions were stopped by addition of 10 μl SDS sample buffer, heated at 95°C for 5 min, and proteins were separated by SDS-PAGE. The gels were fixed in 10% acetic acid for 30 min and dried, and denatured polypeptides were detected by autoradiography.

DNA-joining Assays—Reaction mixtures (5 μl) contained 60 mM Tris-Cl (pH 8.0), 10 mM MgCl₂, 5 mM DTT, 1 μM ATP, 50 μg/ml bovine serum albumin, radioactively labeled polynucleotide substrate (5 ng, 10⁸ cpm), and enzyme fraction (2 μl). The polynucleotide substrates ([5'-32P]poly(dT)poly(dA), [5'-32P]poly(dT)poly(rA)), and [5'-32P]polynucleotide (Oligo(dT)poly(rA)) were prepared as described (11). Reaction mixtures were incubated for 10 min at 37°C. The reaction was stopped by addition of 20 μl of 95% formamide/dye and heating at 80°C for 10 min, and then the mixture was rapidly chilled to 0°C. Ligation products were sepa-
rated on denaturing 20% polyacrylamide gels. The gels were fixed in 10% methanol, 10% acetic acid for 30 min and dried, and ligation products were detected by autoradiography.

Production of Antibodies and Immunoblotting—Histidine-tagged human DNA ligase III recombinant protein was overproduced in Escherichia coli, affinity purified on nickel-agarose, and used to raise rabbit polyclonal antibodies. Antibodies were also raised against synthetic peptides derived from the predicted amino acid sequence of DNA ligase IV and affinity purified by standard procedures (18); peptides were coupled to hemoctyanin, were selected to have an N-terminal cysteine residue, and corresponded to amino acids 526–538 and 834–844 of the 844-residue protein. Proteins were separated by SDS-PAGE (8%), transferred onto nitrocellulose membranes (Schleicher & Schuell), and detected by immunostaining with diluted antibody (1:400). Antigen-antibody complexes were detected by enhanced chemiluminescence using a derivatized secondary antibody (Amersham).

Purification of Mammalian DNA Ligase IV—Frozen HeLa cells (2 × 10^10 cells) were thawed in a hypotonic buffer containing protease inhibitors and disrupted in a Dounce glass homogenizer, and nuclei were collected by low speed centrifugation and a nuclear extract made in the presence of 0.3 M KCl as described by Masutani et al. (19). The nuclear extract was loaded onto a 2.2 × 20-cm phosphocellulose P11 column (Whatman) pre-equilibrated with 20 mM potassium phosphate (pH 7.5), 1 mM EDTA, 1 mM DTT, 10% glycerol, and 150 mM KCl. The column was washed with 2 column volumes of this buffer, and then the protein was eluted with the same buffer containing 400 mM KCl. Crystalline ammonium sulfate was added to the protein solution to 65% saturation, followed by gentle stirring for 30 min. After centrifugation at 10,000 × g for 30 min, the pellets were resuspended in buffer A (50 mM Tris-HCl, 1 mM EDTA, 0.5 mM DTT, 10% glycerol) containing 1 mM NaCl to a protein concentration of approximately 30 mg/ml. The protein was dialyzed for 5 h against the same buffer, and any remaining insoluble material was removed by centrifugation at 10,000 × g for 30 min. The protein supernatant was loaded onto a column (2.5 × 100 cm) of Ultrogel AcA34 (Sepracor/IBF) pre-equilibrated with buffer A containing 1 mM NaCl. Protein fractions eluting from the column were assayed for formation of enzyme-adenylate complexes and subjected to immunoblotting with DNA ligase III-specific antisera and DNA ligase IV-specific antipeptide antibodies. Fractions that were recognized by the DNA ligase IV-specific antibodies were pooled and dialyzed extensively against buffer A containing 25 mM NaCl. The protein was recovered and loaded onto a FPLC Mono S HR5/5 column (Pharmacia Biotech Inv.) pre-equilibrated with buffer A containing 25 mM NaCl. Protein was eluted with a 25–400 mM linear gradient of NaCl before loading onto a FPLC Mono Q HR5/5 column (Pharmacia) pre-equilibrated with buffer A containing 50 mM NaCl. The protein was eluted onto a FPLC Mono Q HR5/5 column (Pharmacia) pre-equilibrated in buffer A containing 50 mM NaCl. Protein was eluted from the column with a 25–400 mM linear gradient of NaCl in buffer A. Fractions that contained protein recognized by the DNA ligase IV-specific antibodies, eluting at 350 mM NaCl, were pooled and dialyzed for 5 h against 50 mM Tris-HCl (pH 7.5, 1 mM EDTA, 0.5 mM DTT, 50% glycerol, and 30 mM NaCl). The protein recovered was aliquoted and frozen at −80 °C or stored at −20 °C. During either method of storage, DNA ligase IV retained full activity for at least 3 months.

RESULTS

Separation of DNA Ligase IV from Other DNA Ligases—An extract of HeLa cell nuclei was partly purified by stepwise phosphocellulose chromatography, concentrated by ammonium sulfate precipitation, and fractionated by gel chromatography in the presence of 1 M NaCl (Fig. 1). Eluted fractions were assayed for enzyme-adenylate formation (Fig. 1A). DNA ligase I occurs as an asymmetric monomer in high salt extracts (20), migrates anomalously slowly during SDS-PAGE, and was apparently present in column fractions 40–42 as a 125-kDa protein (Fig. 1A). Much of the DNA ligase I was removed during preparation of cell nuclei, since the enzyme readily leaches out of nuclei (2). DNA ligase III was present mainly in fractions 37–40 (Fig. 1, A and B). We have observed previously (11) that DNA ligase III elutes before DNA ligase I on gel chromatography, although the two proteins are of similar molecular mass, and this may now be ascribed to the occurrence of DNA ligase III as a salt-resistant heterodimer with the 70-kDa XRCC1 protein (14). Immunoblotting with an antiserum against DNA ligase III (Fig. 1B) verified the presence of DNA ligase III in fractions 37–40 and also identified large, active proteolytic fragments of DNA ligase III, apparently either remaining bound to XRCC1 protein and occurring in fractions 37–40 or eluting as monomers in fractions 45–47. An antibody directed against a C-terminal peptide in DNA ligase IV, not present in either DNA ligase I or III, showed that DNA ligase IV co-chromatographed with DNA ligase III in fractions 37–40. This DNA ligase IV antibody appeared nonspecific, since it did not bind other proteins in the crude fractions (Fig. 1C and D). The identical gel filtration profiles of DNA ligases III and IV indicate that DNA ligase IV also occurs tightly bound to another protein or possibly is present as a salt-resistant homodimer.
DNA ligase IV does not interact with XRCC1 protein (12), so the apparent protein partner of DNA ligase IV is at present unknown.

Further chromatography of pooled AcA34 fractions 36–40 containing DNA ligases III and IV on a Mono S column largely separated the two enzymes (Fig. 2). Activity for enzyme-adenylate formation was mainly present in column fractions 16–18 but was also detected in several fractions eluting earlier (Fig. 2A). Immunoblotting with the DNA ligase III polyclonal antiserum showed that this enzyme was present in fractions 15–20 but not in earlier fractions, although a fragment of DNA ligase III was seen in fractions 14 and 15 (Fig. 2B). Immunoblotting with an antipeptide antibody directed against the C-terminal sequence of DNA ligase IV showed that this enzyme was present in fractions 10–16 (Fig. 2C) and thus accounted for the enzyme-adenylate complexes generated by fractions 10–14 (Fig. 2A). Identical results to those in Fig. 2C, with a slightly weaker signal, were obtained with another antipeptide antibody directed against residues 526–538 of DNA ligase IV, confirming the presence of this protein (data not shown).

Fractions 10–13 of DNA ligase IV from the Mono S column were pooled, passed through a DNA-cellulose column to remove remaining traces and active fragments of DNA ligase III, and then chromatographed on a Mono Q column (Fig. 3). Protein staining of the most active fraction, fraction 10, indicated that DNA ligase IV was the most abundant protein in this fraction, but a few contaminating protein bands were still present (Fig. 3, A and B). Immunoblotting with antisera against DNA ligase III did not reveal detectable amounts of this enzyme in any of the Mono Q fractions (data not shown).

Attempts to overexpress DNA ligase IV cDNA in several E. coli and yeast expression systems under a variety of conditions yielded insoluble protein.

**Joining of Polydeoxynucleotide and Hybrid Substrates by DNA Ligase IV**—The fractions containing DNA ligase IV protein after Mono Q chromatography (Fig. 3, A and B) showed joining activity in a standard DNA ligase assay (Fig. 3C). When the standard reaction mixture was supplemented with 50 mM KCl, DNA ligase IV was ~30% inhibited, whereas DNA ligase III exhibited 2-fold higher activity under these conditions (data not shown).

Different DNA ligases may show different abilities to join single strand breaks in hybrid polynucleotide substrates with one DNA strand and one RNA strand. Thus, DNA ligase I is totally unable to join the oligonucleotides in an oligo(dT)poly(rA) substrate, whereas DNA ligases II and III do so efficiently (21, 11). DNA ligase IV joins this substrate (Fig. 4, A and B), so it is similar to ligase III and different from ligase I in this respect. DNA ligases I and III also can join an oligo(rA)poly(dT) substrate (11), but DNA ligase IV was shown here to be unable to ligate nicks in this polynucleotide (Fig. 4C). Thus, DNA ligase IV shows different substrate specificity from either DNA ligase I or DNA ligase III with regard to its ability to join DNA-RNA hybrids.

DNA ligase IV showed no detectable blunt end joining of plasmid DNA cut with PvuII; DNA ligase III has weak activity in this regard. In contrast, DNA ligase I, as well as T4 DNA ligase, joined this substrate effectively (data not shown). Thus, DNA ligase I remains the best candidate enzyme for DNA blunt end-joining activity in mammalian cells (21).

**Enzyme-Adenylate Formation by DNA Ligase IV**—Northern blotting experiments (12) and the immunoblotting data shown in this article suggest that DNA ligases III and IV are present in similar amounts in cell nuclei as two low abundance proteins. However, in partly purified protein fractions containing both enzymes, the formation of enzyme-adenylate was more efficient with DNA ligase III than with DNA ligase IV (Fig. 2).
physiological function of DNA ligase IV is at present unknown. In enzyme-adenylate formation assays (Fig. 2). The reason that DNA ligase IV was not detected in earlier studies is that the extracyclic adenine amino group of the ATP cofactor (23). The C-terminal domain of the enzyme is involved in a specific protein-protein interaction, since all of the DNA ligase IV present appears to be bound to another factor during gel chromatography (Fig. 1). Identification of this hypothetical partner might give clues to the physiological roles of DNA ligase IV, and analysis of homozygous knockout mice may also be informative with regard to this recently discovered enzyme.

Acknowledgments—We thank Primo Schar and Deborah Barnes for helpful discussions.

REFERENCES

Several distinct DNA ligases have now been found in human cell nuclei, indicating specific roles in DNA metabolism, but the physiological function of DNA ligase IV is at present unknown.

DISCUSSION
DNA ligase IV was identified previously as a unique human cDNA sequence (12). The present data show that an enzyme with the expected antigenic properties of the protein product of this open reading frame can be purified from HeLa cell nuclei, and that the enzyme is able to join phosphodiester bonds in polydeoxyribonucleotide substrates in standard DNA ligation assays. The main structural features of DNA ligase IV are similar to those of other human DNA ligases, with significant sequence homology within the catalytic domain (12). A region with strong sequence homology to the active site of enzyme-adenylate formation in DNA ligase I (22) is readily identified in DNA ligase IV. Both these enzymes, as well as DNA ligase III, have a Glu-Tyr/Thr/Ile-Lys sequence at the active site, where the Lys residue forms a covalent phosphoamide bond with AMP and the side chain of the Glu residue interacts specifically with the extracyclic adenine amino group of the ATP cofactor (23). The reason that DNA ligase IV was not detected in earlier biochemical studies on DNA ligases in mammalian cells is the inability to distinguish between DNA ligases III and IV by SDS-PAGE (Fig. 1) and also the low efficiency of DNA ligase IV in enzyme-adenylate formation assays (Fig. 2).

Several distinct DNA ligases have now been found in human cell nuclei, indicating specific roles in DNA metabolism, but the physiological function of DNA ligase IV is at present unknown.


