A monospecific antibody against calf thymus DNA ligase composed of a single polypeptide with $M_r = 130,000$ cross-reacts with rodent and calf thymus DNA ligases. The antibody precipitates a single $M_r = 200,000$ polypeptide from detergent lysates of $^3$H-leucine-labeled mouse Ehrlich tumor cells and calf thymocytes. Pulse-chase experiments show that the $M_r = 200,000$ polypeptide in Ehrlich tumor cells has a half-life of about 0.5 h. In addition to the $M_r = 200,000$ polypeptide, a $M_r = 130,000$ polypeptide is detected in the partially purified enzyme preparations from radioactive labeled Ehrlich tumor cells. These results suggest that DNA ligase is synthesized in mammalian cells as a $M_r = 200,000$ polypeptide and that the $M_r = 200,000$ polypeptide is degraded to a $M_r = 130,000$ polypeptide by a limited proteolysis in vitro.

DNA ligase (poly(deoxyribonucleotide):poly(deoxyribonucleotide) ligase (AMP-forming) (EC 6.5.1.1)) has been purified from calf thymus, and a monospecific antibody to calf thymus DNA ligase has been prepared by a rabbit immunized with the homogeneous enzyme preparation (1). This enzyme is partially purified by Soderhall and Lindahl (1). A polypeptide is degraded to a $200,000$ polypeptide from detergent lysates of ligases. The antibody precipitates a single polypeptide with a half-life of about 0.5 h and that the $M_r = 200,000$ polypeptide is degraded to a $M_r = 130,000$ polypeptide during the partial purification of DNA ligase.

These results are consistent with our recent observation using electrophoretic blots after SDS-polyacrylamide gel electrophoresis of crude and purified preparations of calf thymus DNA ligase (11).

**Experimental Procedures**

**Chemicals**—All chemicals were the same as described previously (1, 2) unless otherwise specified. IgG-sorb (formalin-fixed Staphylococcus aureus Cowan I strain) was obtained from The Enzyme Center Inc. (Boston, MA). L-[4,5-^3$H$]-Leucine (TRK 683) was from Amer sham (England). RPMI 1640 and fetal bovine serum were products of Gibco (New York). RPMI 1640 without L-glutamine and L-leucine was from Flow Laboratories (Australia). Myosin was obtained from Sigma (St. Louis, MO).

**Cells**—Mouse Ehrlich ascites tumor cells were harvested from the peritoneal cavities of dd albino mice 7 days after inoculation of 0.1 ml of ascites fluid. The cells were washed twice with RPMI 1640 medium lacking leucine. Small pieces of fresh calf thymus obtained from Tokyo Municipal Slaughterhouse were pressed through stainless steel wire mesh (200 gauge). The thymocytes were collected in phosphate-buffered saline containing heparin (10 units/ml) and washed once with RPMI 1640 medium lacking leucine.

**Preparation of IgG Fraction**—IgG fraction was prepared from antisera to calf thymus DNA ligase obtained previously (1) by 35% saturated (NH$_4$)$_2$SO$_4$ precipitation, followed by chromatography on a DEAE-cellulose column (12) equilibrated with 10 mM NaPO$_4$, pH 8.0. The pass-through fraction was treated with 50% saturated (NH$_4$)$_2$SO$_4$, and the precipitate was dissolved in and dialyzed against 10 mM NaPO$_4$, pH 7.5, containing 0.14 M NaCl.

**Biosynthetic Labeling of Cells**—Cells in leucine-deprived RPMI 1640 containing 10% dialyzed fetal bovine serum were metabolically labeled with [H]leucine (120-190 Ci/mmol) in a 5% CO$_2$ incubator at 37 °C. After addition of an excess amount of cold leucine (1.3 mM), cells were either harvested immediately or incubated further for chase. The radiolabeled cells were washed with RPMI 1640 and suspended in 1 ml of ascites fluid. The cells were washed twice with RPMI 1640 medium lacking leucine. The radiolabeled cells were washed with RPMI 1640 and suspended in 10 mM NaPO$_4$, pH 7.5, 0.1 M NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA (9). The lysates were centrifuged for 1 h at 105,000 x g to obtain supernatant solutions for the immunoprecipitation.

**Immunoprecipitation and Fluorography**—The crude extracts and the partially purified DNA ligase from [H]leucine-labeled cells were allowed to react with IgG for 12 h at 0 °C. The immune complex was precipitated with formalin-fixed S. aureus according to the method of Kessler (13). The precipitates were washed at least 6 times with detergent-lysis buffer, suspended in sample buffer containing 20 mM NaPO$_4$, pH 7.5, 0.1 M NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA (9). The lysates were centrifuged for 1 h at 105,000 x g to obtain supernatant solutions for the immunoprecipitation.

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labeled Ehrlich tumor cells mixed with 0.3 g (weight) of nonlabeled Ehrlich cells were homogenized in 3 ml of 20 mM KPO₄, pH 7.5, 0.5 M KCl, 0.5% Triton X-100, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM EDTA with a glass-Teflon homogenizer, followed by sonication with a Branson sonifier equipped with a microprobe (15 s × 2). After a 1-h centrifugation at 105,000 × g, the resulting supernatant solution was diluted by addition of 5 volumes of 10 mM KPO₄, pH 7.5, 0.5 mM dithiothreitol, 0.1 mM EDTA (Buffer A). The solution was applied to a phosphocellulose column (1.4 × 10 cm) equilibrated with Buffer A and washed with 16 ml of Buffer B. The enzyme was eluted with a linear KPO₄ concentration (0-1 M) in 80 ml of Buffer A. Active fractions (4 ml/fraction) were combined and applied to a hydroxylapatite column (1 × 4 cm) equilibrated with Buffer A containing 0.5 M KCl and 10% glycerol. After being washed with 6 ml of the above buffer, the enzyme was eluted with a linear KPO₄ concentration (10 mM to 0.4 M) in 30 ml of KPO₄ buffer (pH 7.5) containing 0.5 M KCl, 0.5 mM dithiothreitol, 0.1 mM EDTA, and 10% glycerol.

**Assay for DNA Ligase Activity**—DNA ligase activity was determined as described previously (1, 2). One unit of DNA ligase was defined as the amount converting 1 nmol of ³²P to an alkali phosphatase-resistant form/min under the standard assay conditions.

**Protein Determination**—Protein was determined by the method of Bradford (16).

**RESULTS**

**Immunoprecipitation of a Mᵦ = 200,000 Polypeptide by Anti-(DNA ligase)IgG**—As described previously (1), immunoglobulin fractions of rabbit anti sera to calf thymus DNA ligase react with the enzyme in an immunodiffusion test and neutralize mammalian DNA ligase activity. IgG from the antisera completely inhibited the activity of calf thymus and rodent DNA ligases, but control IgG from nonimmune rabbit sera did not. Using formalin-fixed *S. aureus* for the immunoprecipitation, 1 milligram of calf thymus DNA ligase was completely neutralized with 6 μg of anti-IgG, and the titer of the IgG for the enzyme from mouse Ehrlich tumor cells was about one-fifth that for calf thymus DNA ligase (see Ref. 1).

Mouse Ehrlich tumor cells containing a high level of DNA ligase activity (1, 2) were metabolically labeled with [³H]leucine. The radiolabeled cells were lysed with lysis buffer containing three detergents (9), the lysates were incubated with anti-(DNA ligase)IgG, and the resulting antigen-antibody complex was collected by binding to *S. aureus* cells. The precipitates were analyzed by SDS-polyacrylamide gel electrophoresis, followed by fluorography. A Mᵦ = 200,000 polypeptide was observed when incubated with anti-IgG (Fig. 1, Lane A-1) but not when incubated with control IgG (Lane A-3).

Using calf thymocytes instead of mouse Ehrlich tumor cells, a Mᵦ = 200,000 polypeptide was also detected with anti-IgG (Fig. 1, Lane B-1). The other polypeptides observed in the samples treated with control IgG as well as with immune IgG (Fig. 1) would be nonspecific ones, because only a Mᵦ = 200,000 polypeptide band observed in Ehrlich tumor cells and calf thymocytes disappeared by addition of an excess amount of DNA ligase purified from calf thymus (Fig. 1, Lanes A-2 and B-2). Purified DNA ligases from calf thymus and Ehrlich tumor cells are composed of a single polypeptide of Mᵦ = 120,000-130,000 (1, 2). In the biosynthetic experiments, however, a similar size of polypeptide could not be detected (Fig. 1).

Therefore, the Mᵦ = 200,000 polypeptide seems to be synthesized in mammalian cells as DNA ligase enzyme. This is consistent with our recent observation (11) suggesting that a Mᵦ = 200,000 polypeptide, a native form of DNA ligase present in cells, is extraordinarily susceptible to a limited digestion in vitro.

**Relationship between a Mᵦ = 200,000 Polypeptide and DNA Ligase Enzyme**—To examine whether the radioactive Mᵦ = 200,000 polypeptide is co-purified with DNA ligase activity, DNA ligase was purified from the extract of [³H]leucine-labeled Ehrlich tumor cells by phosphocellulose column chromatography (data not shown) and hydroxylapatite column chromatography (Fig. 2A). DNA ligase activity was eluted as a single peak at a KPO₄ concentration of about 0.1 M. Immunoprecipitation of the enzyme fraction (Fig. 2A, Fraction I) showed a Mᵦ = 130,000 polypeptide band as well as a Mᵦ = 200,000 one (Fig. 2B, Lane I-1). The Mᵦ = 130,000 polypeptide is likely a degradative product from a Mᵦ = 200,000 polypeptide during the partial purification of DNA ligase from Ehrlich tumor cells. No specific polypeptide was precipitated by IgG from the fraction containing no DNA ligase activity (Fig. 2B, Lanes II-3 and -4).

**Pulse-Chase Experiments**—To estimate a half-life of the Mᵦ = 200,000 polypeptide, Ehrlich tumor cells were pulsed with [³H]leucine for 5 h and then chased for 1, 2, or 4 h in the medium containing an excess amount of unlabeled leucine. Immunoprecipitation of the cell lysates was carried out with anti-IgG. As shown in Fig. 3, the intensity of the Mᵦ = 200,000 polypeptide band decreased progressively. The half-life of the Mᵦ = 200,000 polypeptide was estimated about 0.5 h.

**DISCUSSION**

Immunoprecipitation techniques with monospecific antibodies have been successfully utilized to investigate the biosynthetic process of murine and human terminal deoxynucleotidyltransferases (9, 17) and chick embryo DNA polymerase β (10). Using this immunoprecipitation technique and a monospecific antibody to calf thymus DNA ligase, we have studied the synthesis of DNA ligase in [³H]leucine-labeled mouse Ehrlich tumor cells and calf thymocytes. We found that only a Mᵦ = 200,000 polypeptide is specifically immunoprecipitable from metabolically labeled culture and primary cells (Fig. 1). Both a Mᵦ = 200,000 polypeptide and DNA
FIG. 2. Co-purification of DNA ligase activity and immunoprecipitable polypeptides on hydroxylapatite chromatography. Ehrlich tumor cells \( (3 \times 10^7 \text{ cells}) \) were labeled with 0.15 mCi of \( [\text{H}]\)leucine for 5 h, and then cell extract was prepared for chromatography as described under "Experimental Procedures." The active fractions of phosphocellulose column chromatography were combined and applied to a hydroxylapatite column (A). Acid-precipitable \( ^3\text{H} \) radioactivity in 30-\( \mu \)l aliquots of fractions (○—○) and DNA ligase activity in 10-\( \mu \)l aliquots (●—●) were determined. B, pooled fractions I and II in A) were dialyzed overnight against 60% saturated \((\text{NH}_4)_2\text{SO}_4\), \( 20 \text{ mM KPO}_4, \text{pH 7.5}, 1 \text{ mM EDTA}, 0.5 \text{ mM phenylmethylsulfonyl fluoride}, 0.5 \text{ mM dithiothreitol.} \) The precipitates were dissolved in and dialyzed against 20 mM Tris-HCl, pH 7.8, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 10% glycerol. Proteins containing acid-precipitable \( ^3\text{H} \) radioactivity from I and II fractions were treated with 1.2 mg of anti-(DNA ligase)IgG (Lanes 1 and 3) or control IgG (Lanes 2 and 4). The immunoprecipitable proteins were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography as described under "Experimental Procedures." The fluorogram was obtained after 1-month exposure.

FIG. 3. Pulse-chase labeling of Ehrlich tumor cells. Ehrlich tumor cells \( (3 \times 10^7 \text{ cells/dish}) \) were incubated for 5 h in 15 ml of leucine-deprived RPMI 1640 containing 10% dialyzed fetal bovine serum and 0.125 mCi of \( [\text{H}]\)leucine. After addition of 1.3 mM unlabeled leucine to each dish, the cells were further incubated for 0 h \( (\text{Lane 1}) \), 1 h \( (\text{Lane 2}) \), 2 h \( (\text{Lane 3}) \), and 4 h \( (\text{Lane 4}) \). Cells were extracted for immunoprecipitation with 350 \( \mu \)g of anti-(DNA ligase)IgG. Immunoprecipitable proteins were subjected to SDS-polyacrylamide gel electrophoresis, followed by fluorography. Exposure was for 16 days.

DNA ligase enzyme activity in Ehrlich tumor cells were eluted from hydroxylapatite column at about 0.1 mM KPO4, and co-immunoprecipitated with the antibody (Fig. 2). In addition to the \( M_t = 200,000 \) polypeptide in the partially purified fraction on hydroxylapatite column chromatography, a \( M_t = 130,000 \) polypeptide was observed (Fig. 2B, Lane I-1). Polypeptides with \( M_t = 120,000-130,000 \) have been reported in the purified DNA ligase (1) and in the highly purified enzyme from Ehrlich tumor cells (2). Recently, we have suggested that mammalian DNA ligase exists \textit{in vivo} as an enzyme form composed of a single polypeptide with \( M_t = 200,000 \) and that the \( M_t = 200,000 \) polypeptide is easily degraded to a \( M_t = 130,000 \) polypeptide and then to a \( M_t = 90,000 \) one during the isolation and purification procedures (11). Therefore, a radiolabeled polypeptide with \( M_t = 130,000 \) (Fig. 2B) is most likely to be a degradative product of the \( M_t = 200,000 \) polypeptide during the partial purification of DNA ligase from Ehrlich tumor cells.

Pulse-chase experiments indicated that a half-life of the \( M_t = 200,000 \) polypeptide is about 0.5 h in Ehrlich tumor cells (Fig. 3). The half-life of DNA ligase is extremely short in comparison with that of chick embryo DNA polymerase \( \beta \) (half-life = about 10 h) (10). Murine terminal deoxynucleotidyltransferase has also a long half-life of less than 10 h in cultured thymoma cell lines. The short half-life of DNA ligase is probably ascribed to the following reason. DNA ligase is an inducible enzyme in correlation with DNA synthesis (18-25), whereas DNA polymerase \( \beta \) and terminal transferase are likely constitutive enzymes. For instance, DNA ligase activity in regenerating rat liver reaches maximal level about 24 h after partial hepatectomy in correlation with the increase in DNA synthesis (19). When cycloheximide was administered to rats 24 h after the operation to prevent syntheses of protein and DNA, DNA ligase activity decreased with an apparent half-life of 1-1.5 h (19). This value is comparable to the half-life of about 0.5 h estimated from pulse-chase experiments of DNA ligase in mouse Ehrlich tumor cells. Since DNA ligase is one of the important enzymes induced in the period of increased DNA synthesis, it is surely a useful tool for investigation on the regulation of DNA synthesis.

Two distinct forms of DNA ligase have been reported to exist in mammalian cells and tissues (3-7). Söderhäll (20) demonstrated that in regenerating rat liver DNA ligase I activity increases but DNA ligase II remains constant. Alter-
natively, DNA ligase II with a lower molecular weight and a higher $K_m$ for ATP may be an artifact derived from DNA ligase I (7, 8). When we can obtain antibodies to DNA ligase II, it would be possible to determine whether DNA ligase II is present in vivo or an artifact. Our antibody to calf thymus DNA ligase I partially purified by Söderhäll and Lindahl (3, 4) did not inhibit calf thymus DNA ligase II.2

REFERENCES

1 H. Teraoka and K. Tsukada, unpublished data.