Purification of DNA Ligases from Mouse Testis and their Behavior during Meiosis.

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ABSTRACT. Two types of DNA ligase, I and II, have been purified approximately 4,000-fold from mouse testes and 500-fold from nuclei of mouse spermatocytes. DNA ligase I and II consisted of single polypeptides with molecular weights of 95,000 and 65,000, respectively, according to the estimation by SDS-polyacrylamide gel electrophoresis and the AMP-binding assay. Ligase activities were higher in premeiotic spermatogonia and spermatocytes than those in liver and bone marrow cells. Moreover, DNA ligase II showed rapid increase during meiotic prophase and a decrease in round spermatids. Since this behavior of DNA ligase II is consistent with that of m-rec and DNA polymerase γ, both of which have been shown to be involved in DNA recombination in meiotic cells, DNA ligase II might be an enzyme which works at the final step of meiotic recombination reaction.

DNA ligase (EC 6.5.1.1) is an enzyme which catalyzes the formation of the phosphodiester bond between adjacent 5'-phosphoryl and 3'-hydroxyl groups using nicked duplex DNA or two blunt-ended duplex DNA as substrates. In prokaryotic cells, only one type of DNA ligase exists, and this enzyme is involved in the DNA metabolisms, e.g., replication, recombination, and repair (for reviews, see Refs. 1, 2), which are essential for proliferation and/or survival of cells. In mammalian cells, two distinct types of DNA ligase, I and II, have been discovered so far (3). These two enzymes were purified from calf thymus and it was shown that both DNA ligase I and DNA ligase II consist of single polypeptides with molecular weights of 130,000 and 68,000, respectively (4, 5). DNA ligase I has a lower binding affinity to DNA than DNA ligase II does (3-5). DNA ligase I appeared to be closely associated with the DNA replication (3, 6, 7), while DNA ligase II is associated with the DNA repair system (3, 7, 8).

In meiotic cells, repair synthesis and subsequent recombination of DNA have been reported to take place during the prophase period (9, 10). A number of proteins which might be involved in this recombination have been purified and characterized (9, 11). In this study, partial purification of two types of DNA ligase from mouse spermatocytes was carried out, and the activity profiles of these enzymes in the meiotic process were observed in order to investigate the roles of DNA ligases in the recombination reaction during meiosis.

MATERIALS AND METHODS

Chemicals. [γ-32P] ATP was purchased from the Radiochemical Centre (Amersham, England). DEAE-cellulose (DE52) and phosphocellulose (P11) were obtained from Whatman. Butyl-Toyopearl 650 and Toyopearl HW55 were obtained from Toso (Tokyo, Japan). Hydroxyapatite gel was purchased from Toa-nenryo Kogyo KK (Tokyo, Japan). DNA-cellulose (native DNA and denatured) were obtained from Sigma Chemical Co.

Separation of meiocytes from testes and isolation of somatic materials. Random-bred Swiss albino male mice were the source of spermatocytes and somatic tissues. The spermatogenic cells were fractionated by the procedure of Chandley et al. (12) using bovine serum albumin (BSA) gradient in staphut. Cells and tissues were frozen in liquid nitrogen immediately after isolation and maintained at -80°C until use. In some cases, testes were purchased through MBL Co., Nagoya.

Assay for DNA ligase activity. The activity of DNA ligase was tested by the ability of joining pUC19 DNA which had been digested to a linear form by endonuclease Pst I. The standard reaction mixture (15 μl) contained 20 mM Tris-HCl(pH 7.5), 10 mM MgCl2, 1 mM ATP, 5 mM DTT, 0.2 μg of pUC19 linear DNA, and the enzyme fraction containing 0.1-1.0 μg of protein. After incubation for 30 min at 30°C,
the reaction was terminated by adding 3 μl of stop mixture containing 2% (w/v) sucrose, 0.4% bromophenyl blue, 4% NaDodSO₄, 50 mM EDTA, and proteinase K at a final concentration of 0.1 mg/ml. This mixture was incubated at 42°C for 10 min and then loaded onto a 0.9% agarose gel. After the electrophoresis, the gel was stained by 0.5 μg/ml of ethidium bromide and the DNA bands were observed under ultraviolet light. One unit of activity was defined as the amount of DNA ligase necessary to join 50% of the substrate DNA molecule (0.2 μg) to a catemer form measured by using a Cosmo Bio densitometer analyzer at 30°C for 30 min.

Purification of DNA ligase I. All operations were performed at 0-4°C unless otherwise indicated. Testes removed from freshly sacrificed mice were stored at −80°C until use. The frozen testes (100g) were crushed into fine powder in liquid nitrogen by using a porcelain mortar and pestle, and homogenized with 450 ml of 20 mM Tris-HCl (pH 7.5), 0.2 mM EGTA, 0.1 mM PMSF, 5 mM DTT, and 10% glycerol (Buffer A). This homogenate was passed through two layers of gauze. After the addition of NaCl to a final concentration of 1.0 M, the homogenate was centrifuged at 200,000 × g for 1 h. The supernatant was filtered through a 3MM paper filter to remove lipidlike material and the crude extract was obtained. Ammonium sulfate was added to the crude extract to a final concentration of 35% saturation and the precipitate was discarded after the centrifugation. The supernatant fluid was adjusted to 75% saturation of ammonium sulfate and the precipitate was collected by centrifugation. The resulting precipitate was dissolved in buffer A containing 0.4 M NaCl (step 2). The solution was then applied to a DEAE-cellulose column and washed with 0.4 M NaCl in buffer A. DNA ligase I was recovered in the pass-through fraction (step 3). This fraction was dialyzed overnight against buffer A containing 10% polyethylene-glycol #20,000, and then applied again to a DEAE-cellulose column equilibrated with buffer A. The protein was eluted with NaCl at concentrations between 0.03 M and 0.05 M (step 4). The active fraction was directly applied to a native DNA-cellulose column equilibrated with buffer A. The protein was eluted with NaCl at concentrations between 0.03 M and 0.05 M (step 4). The active fraction was directly applied to a native DNA-cellulose column equilibrated with buffer A. The fraction was then applied to Butyl-TOYOPAL column BT650M and washed with the same buffer containing 33% ammonium sulfate. DNA ligase II was eluted at 15% ammonium sulfate concentration, and fractions with high enzyme activity were pooled (step 4). The pooled fraction was dialyzed against buffer A for 4 hours, then applied to a native DNA-cellulose column. After washing with buffer A, proteins bound to the column were eluted with NaCl at concentrations between 0.35 M and 0.45 M (step 5). The active fractions were pooled and dialyzed against buffer K containing 10% polyethylene glycol #20,000, and then applied to a hydroxyapatite column equilibrated with buffer K. After the column was washed with buffer K, ligase II was eluted with KPO₄ at a concentration of 0.38 M (step 6). The active fraction was concentrated by using centrifuge 30, and applied to a gel filtration column, HW55, which was preequilibrated with 0.15 M NaCl in buffer A. The fractions with significant activities were combined and dialyzed overnight against buffer A containing 10% polyethylene glycol #20,000 (step 7).

Formation of DNA ligase-adenylate. A covalent ligase-[³²P] adenylyl complex (ligase-AMP) was formed in a 20 μl reaction mixture containing 30 mM Tris-HCl (pH 7.5), 15 mM MgCl₂, 1 mM DTT, 100 μM [α-³²P] ATP (1.2 × 10⁸ cpm/nmole), and the enzyme fraction (13, 14). After incubation at 30°C for 60 min, the reaction was terminated by addition of 0.5 M EDTA to 25 mM. The solution was applied to a Biogel P6 spin column (75 μl in a 0.5 ml eppendorf tube) and was centrifuged at 800 rpm for 1 min. Then SDS-polyacrylamide gel electrophoresis was carried out on a slab gel with
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3% stacking gel according to the methods of Laemmli (15). The gel was fixed and stained with Coomassie brilliant blue, and autoradiography was carried out with Fuji X-ray film RXO-H.

RESULTS

Purification of DNA ligase I and II. The procedures and the results of purification are summarized in Tables I and II. The overall purification of ligase I was about 4000-fold with a yield of 5.0% and DNA ligase II about 500-fold from nuclear extracts with a yield of 13%. DNA ligase II was effectively separated from DNA ligase I by using purified nuclei as the starting material and by native DNA cellulose column chromatography. DNA ligase II has been called "the nuclear ligase" and the affinity to single-strand and duplex DNA is much higher than that of DNA ligase I (3, 5). In this course of purification of mouse testes, DNA ligase II was not released from nuclei during the process of nuclear fractionation; indeed it bound more tightly to the native DNA-cellulose column than DNA ligase I did.

Enzymatic purity and molecular properties. Figure 1 shows the SDS-polyacrylamide gel patterns of the final fractions of DNA ligase I and II. As shown in lanes 1 and 3, major protein bands with molecular weights of 95,000 and 65,000, respectively, were observed for DNA ligase I and II on the stained gel. For confirmation of these bands as DNA ligases, the binding ability with 32P-AMP was tested according to the methods of Söderhäll and Lindahl (13), and Rabin, et al. (14). An obvious band was seen at the position of a molecular weight of 95,000 for DNA ligase I-[32P] AMP complex and 65,000 for ligase II-[32P] AMP complex (Fig. 1). Judging from the molecular weights of the enzymes previously reported (3-5) and the difference of the affinity to [32P] ATP (data not shown), we concluded that each of these proteins corresponded to DNA ligase I and II. One faintly stained band with a molecular weight of 70,000 in the ligase II fraction (Fig. 1 lane 3) did not form a complex with 32P-AMP.

We often observed minor bands with molecular weights of 130,000, 72,000, 45,000, and 40,000 in the DNA ligase I fraction, which were not seen in Fig. 1. Mezzina, et al. (16, 17), Teraoka, et al. (4), and Teraoka and Tsukada (18) reported that these proteins were the degradation products of DNA ligase I having an original molecular weight of 200,000. Our results confirmed their reports since these minor bands did form complexes with 32P-AMP, as is shown in Fig. 3.

Behavior of DNA ligase I and II in meiotic process. It had been reported that frequent recombination be-

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<th>Table I. Purification of DNA ligase I from mouse testes.</th>
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<td>Purification step</td>
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<td>1. Crude extract</td>
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<td>2. Ammonium sulfate</td>
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<td>3. DEAE-cellulose (I)</td>
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<td>5. DNA-cellulose (native)</td>
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<td>6. Hydroxyapatite</td>
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<td>7. Phosphocellulose (P11)</td>
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<th>Table II. Purification of DNA ligase II from mouse spermatocytes.</th>
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<td>Purification step</td>
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<tr>
<td>1. Nuclear extract (EP, LP)</td>
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<tr>
<td>2. Ammonium sulfate</td>
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<td>3. DEAE-cellulose</td>
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<td>4. Butyltoyopearl BT650</td>
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<td>5. DNA-cellulose (native)</td>
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<td>6. Hydroxyapatite</td>
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<td>7. Toyopearl HW55</td>
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Fig. 1. SDS-polyacrylamide gel electrophoresis of DNA ligases I and II. The purified DNA ligase-[32P] AMP complexes were electrophoresed on an 11% SDS-polyacrylamide gel overlayed by a 3% stacking gel. The gels stained with Coomassie blue are shown in lanes 1 and 3, and autoradiograms are shown in lanes 2 and 4. Lanes 1 and 2: 200 ng of the DNA ligase I fraction after step 8 in Table I. Lanes 3 and 4: 100 ng of the DNA ligase II fraction after step 7 in Table 2.
between homologous chromosomes occurs during the pro-
phase of meiotic division (9). To investigate whether
DNA ligases play any role in the final step of this event,
the entire activity of DNA ligases was traced over the
period of premeiotic to round spermatid. The spermatocyte
cells were fractionated by staphut as described
earlier (12) and separated into three groups, i.e., early
prophase (EP), late prophase (LP), and round sper-
matids (RT), which confined the cells in the premeiotic
S-phase to a population of no more than 17% of the
total cells. Premeiotic cells (Pre-P), which were free of
round spermatids, were obtained from testes of
prepubertal mice. This fraction may have been con-
taminated with leptotene cells but not with cells of any
later stages. The results were plotted in Fig. 2. A high
level of ligase activity was detected in the cells from
premeiotic S-phase (Pre-P) to late prophase (LP). The
activity level decreased in postmeiotic cells (RT), but
remained at a higher level than those of liver and bone
marrow cells.

In order to estimate the contribution of either of
DNA ligase I or II to the whole ligase activity detected
in meiocytes, the following experiment was carried out.
Ligase-[32P] AMP complexes previously formed with
the cell extracts from the several meiotic stages were
separated on SDS-polyacrylamide gel electrophoresis
and the positions of the ligases were detected by auto-
radiography so that the behavior of DNA ligase I and II
could be separately observed. As shown in Fig. 3,
several bands corresponding to the proteins which
bound to labeled nucleotides were identified. These
bands completely disappeared when substrate DNA was
added to the reaction mixture after complex formation,
indicating that the complexes originated from specific
DNA ligase-[32P] AMP binding (data not shown). The
amount of DNA ligase II (MW 65,000) increased signifi-
cantly when spermatocytes entered the prophase of
meiosis, and decreased to an undetectable level in round
spermatids. Meiotic recombination is known to take
place during prophase, and m-rec and DNA polymerase
β which have previously been reported to be involved in
meiotic recombination (9, 19), showed an activity pro-
file similar to that of DNA ligase II, which strongly sug-
gests that DNA ligase II plays some role in this reaction.
DNA ligase I, on the other hand, was detected
throughout the meiotic process except for the RT
period where a residual amount of DNA ligase I,

![Fig. 2. Profile of DNA ligase activity during spermatogenesis. DNA ligase activity was assayed in spermatocyte extracts at premeiotic S-phase (Pre-P), early prophase (EP), late prophase (LP) and the postmeiotic stages of round spermatids (RT). The somatic cells were obtained from liver (L) and bone marrow (BM). In this experiment, DNA ligase was partially purified as described below. The crude homogenates of spermatocyte or somatic cells were applied to a native DNA-cellulose column after removal of nucleic acids by DEAE-cellulose column chromatography. After the column was washed with buffer A (described in Materials and Methods), the DNA ligase fraction was eluted with 0.5 M NaCl in buffer A. The error bar indicates the distribution of three independent measurements, and the average is shown by the symbols.](image)

![Fig. 3. DNA ligase-[32P] AMP complexes from extracts of spermatocyte cells. The DNA ligase-[32P] AMP complexes from extracts of spermatocyte cells at different stages were electrophoresed on an 11% SDS-polyacrylamide gel overlaid by a 3% stacking gel. The gels were exposed to X-ray film and the complexes were detected by autoradiography. Lane 1: Pre-P fraction, lane 2: EP fraction, lane 3: LP fraction and lane 4: RT fraction. The abbrevations are the same as in Fig. 1.](image)
originating from the contaminated Pre-P cells, was seen. However, the large molecule of MW 130,000 only existed in the extract from preprophase, and the molecular weight of the major protein decreased as the meiosis proceeded.

**DISCUSSION**

In this study, two distinct types of DNA ligase, having molecular weight of 95,000 and 65,000, respectively, have been partially purified from mouse testes. In previous studies, the presence of two types of DNA ligase was reported in mammalian cells and/or tissues (3–8, 13, 16–18). These two DNA ligases, I and II, were shown to differ by their chromatographic behavior, immunological responses and molecular properties (3–5, 8, 18), though some experimental data pointed out the possibility that DNA ligase II may be a secondary product of DNA ligase I (20,21). Comparing our results with those in the previous reports and judging from the criteria described therein, it is reasonable at present to correspond a MW 95,000 protein from mouse testes to the degraded but active form of DNA ligase I and a MW 65,000 protein to DNA ligase II. Analyses using specific antibodies against cloned genes of the respective enzymes would facilitate further identification of these two enzymes.

In this study with testes, we observed that DNA ligase I with a high molecular weight predominantly existed during the premeiotic S-phase and rapidly decreased when the spermatocytes entered the meiotic prophase (Fig. 3). It has been suggested that DNA ligase I connects the Okazaki short pieces during DNA replication whereas DNA ligase II seals the final gaps in the DNA repair reaction (3, 7, 8). It is therefore plausible that DNA ligase I, which was active in DNA replication reaction during the premeiotic S-phase, was degraded immediately after the cell proceeded to the meiotic prophase. DNA ligase II, on the other hand, was shown to exist predominantly during meiotic prophase in mouse spermatocytes (22). This profile of activity is similar to those of meiotic specific proteins called m-rec and recombinase, which probably catalyze meiotic recombination (9), and of DNA polymerase β, the enzyme which is considered to function in repair and recombination reactions in both somatic and meiotic cells (19). Thus, DNA ligase II is most likely to be the enzyme which catalyzes the DNA ligation at the final step in the meiotic recombination process.

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