

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

Atsushi Higashitani, Satoshi Tabata, Hideki Endo* and Yasuo Hotta†

Biological Institute, School of Science, Nagoya University, Nagoya 464-01, Japan

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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 β , 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. [α -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 staput. 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 MgCl_2 , 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 ,

† To whom correspondence should be addressed.

* Present address: Institute for Chemical Research, Kyoto University, Uji, Kyoto-Fu 611, Japan

Abbreviations used: MW, molecular weight; EGTA, ethyleneglycol bis (β -amino-ethylether)-N,N',N'-tetraacetic acid; EDTA, ethylenediamine tetraacetic acid; PMSF, phenyl-methyl sulfonyl fluoride; DTT, dithiothreitol; ATP; adenosine triphosphate, AMP; adenosine monophosphate; SDS, sodium dodecylsulfate.

the reaction was terminated by adding 3 μ l 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 μ g/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 μ g) to a catemer form measured by using a Cosmo Bio densito-pattern 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 mouse 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 $\times$ 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 0.05 M NaCl in buffer A. After the column was washed with the same buffer, the enzyme was eluted with a linear NaCl gradient (0.05 M to 0.6 M) in buffer A. The peak of the eluted enzyme activity appeared at around 0.3 M NaCl. Active fractions were pooled and concentrated by a centricon 30 (Amicon Co. LTD.), and the medium was changed to buffer K (20 mM KPO₄ (pH 7.5), 5 mM DTT, 0.2 mM EGTA, 0.1 mM PMSF, and 10% glycerol) (step 5). The enzyme solution was applied to a hydroxyapatite column equilibrated with buffer K. Proteins which bound to hydroxyapatite were eluted with 0.15 M KPO₄ (step 6). After the dialysis against buffer K, the enzyme fraction was applied to a phosphocellulose (P-11) column equilibrated with buffer K containing 0.1 M KCl. After the column was washed with the same buffer, the enzyme was eluted with KCl at concentrations between 0.35 M and 0.45 M (step 7). The active fraction was concentrated by the centricon 30 and applied to a gel filtration HW55 column

equilibrated with 0.15 M NaCl in buffer A. The major active fractions were combined and dialyzed overnight against 300 ml of buffer A containing 10% polyethylene-glycol 20,000 (step 8).

Purification of DNA ligase II. The spermatogenic cells fractionated from testes by using the staput procedure (12) were stored at –80°C until use. The frozen spermatocytes (40 g) were crushed into fine powder in liquid nitrogen by using a porcelain mortar and a pestle and suspended in buffer A containing 0.3 M sucrose and 10 mM MgCl₂. After the mixture was homogenized again by polytron and filtrated through two layers of gauze, the homogenate was centrifuged at 800 $\times$ g for 20 min. The nuclear pellet was washed twice with the same sucrose buffer. The nuclei were precipitated through buffer A containing 2.3 M sucrose by centrifugation at 150,000 $\times$ g for 1 h and resuspended in buffer A containing 1.0 M NaCl. The viscous solution was centrifuged at 200,000 $\times$ g for 1 h to obtain the nuclear extract. The ammonium sulfate precipitation step (step 2) and DEAE-cellulose column (step 3) were applied according to the purification procedure for DNA ligase I. Solid ammonium sulfate was added the pass-through fraction from the DEAE-cellulose column to a final 75% saturation. After precipitation by standing on ice overnight, the precipitate was collected by centrifugation. The precipitate was dissolved in buffer A containing ammonium sulfate to 33% saturation. The enzyme fraction was then applied to Butyltoypearl 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% polyethyleneglycol #20,000, 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 centricon 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% polyethyleneglycol #20,000 (step 7).

Formation of DNA ligase-adenylate. A covalent ligase-[³²P] adenylate complex (ligase-AMP) was formed in a 20 μ l reaction mixture containing 30 mM Tris-HCl (pH 7.5), 15 mM MgCl₂, 1 mM DTT, 100 μ M [α -³²P] ATP (1.2 $\times$ 10⁷ 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 μ l 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

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 ^{32}P -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- ^{32}P AMP complex and 65,000 for ligase II- ^{32}P AMP complex (Fig. 1). Judging from the molecular weights of the enzymes previously reported (3-5) and the difference of the affinity to ^{32}P 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 ^{32}P -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 ^{32}P -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-

Table I. PURIFICATION OF DNA LIGASE I FROM MOUSE TESTES.

Purification step	Total protein	Total activity	Specific activity	Yield
	mg	units	units/mg	%
1. Crude extract	500	36000	72.0	100
2. Ammonium sulfate	362	30000	82.9	83
3. DEAE-cellulose (I)	277	30000	108	83
4. DEAE-cellulose (II)	91	30000	330	83
5. DNA-cellulose (native)	9	27000	3000	75
6. Hydroxyapatite	3	16000	5333	44
7. Phosphocellulose (P11)	0.1	4000	40000	11
8. Toyopearl HW55	0.006	1800	300000	5

Table II. PURIFICATION OF DNA LIGASE II FROM MOUSE SPERMATOCYTES.

Purification step	Total protein	Total activity	Specific activity	Yield
	mg	units	units/mg	%
1. Nuclear extract (EP, LP)	8.0	960	120	100
2. Ammonium sulfate	6.2	960	155	100
3. DEAE-cellulose	2.4	730	304	76
4. Butyltoyopearl BT650	0.2	390	1950	41
5. DNA-cellulose (native)	0.03	330	11000	34
6. Hydroxyapatite	0.005	210	42000	22
7. Toyopearl HW55	0.002	120	60000	13

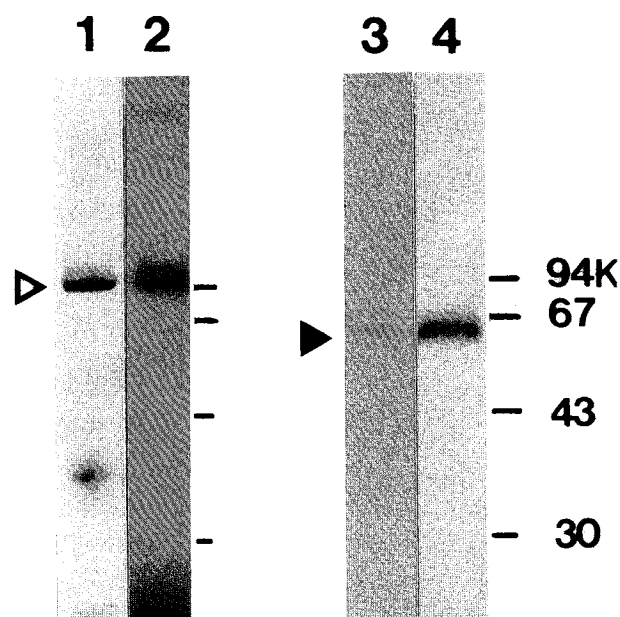


Fig. 1. SDS-polyacrylamide gel electrophoresis of DNA ligases I and II. The purified DNA ligase- ^{32}P AMP complexes were electrophoresed on an 11% SDS-polyacrylamide gel overlaid by a 3% stacking gel. The gels stained with Coomassie blue are shown in lanes 1 and 3, and autoradiograms are shown in lanes 2 and 4. Lanes 1 and 2: 200 ng of the DNA ligase I fraction after step 8 in table 1. Lanes 3 and 4: 100 ng of the DNA ligase II fraction after step 7 in Table 2.

tween homologous chromosomes occurs during the prophase 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 staput as described earlier (12) and separated into three groups, *i.e.*, early prophase (EP), late prophase (LP), and round spermatids (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 contaminated 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- ^{32}P AMP complexes previously formed with the cell extracts from the several meiotic stages were separated on SDS-polyacrylamide gel electrophoresis

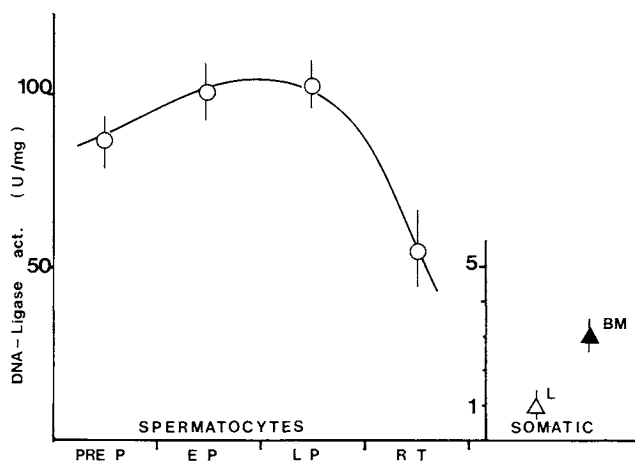


Fig. 2. Profile of DNA ligase activity during spermatogenesis. DNA ligase activity was assayed in spermatocyte extracts at premeiotic S-phase (Pre-P), early prophase (EP), late prophase (LP) and the postmeiotic stages of round spermatids (RT). The somatic cells were obtained from liver (L) and bone marrow (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.

and the positions of the ligases were detected by autoradiography 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- ^{32}P AMP binding (data not shown). The amount of DNA ligase II (MW 65,000) increased significantly 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 profile similar to that of DNA ligase II, which strongly suggests 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,

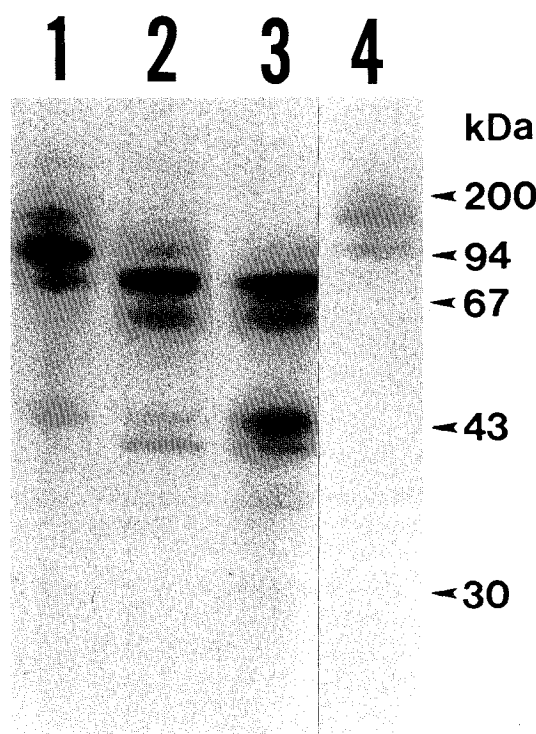


Fig. 3. DNA ligase- ^{32}P AMP complexes from extracts of spermatocyte cells. The DNA ligase- ^{32}P AMP complexes from extracts of spermatocyte cells at different stages were electrophoresed on an 11% SDS-polyacrylamide gel overlaid by a 3% stacking gel. The gels were exposed to X-ray film and the complexes were detected by autoradiography. Lane 1: Pre-P fraction, lane 2: EP fraction, lane 3: LP fraction and lane 4: RT fraction. The abbreviations are the same as in Fig. 1.

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 and 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 division. DNA ligase II, on the other hand, was shown to exist predominantly during meiotic prophase in mouse spermatocytes (Fig. 3), as had been reported in ram 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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