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Two Types of DNA Ligase I Activity in Lymphoblastoid Cells from Patients with Bloom's Syndrome

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DNA ligases I and II were separated by hydroxylapatite (HA) column chromatography in cell-free extracts of lymphoblastoid cell lines (LCLs) derived from two unrelated patients with Bloom's syndrome (BS) and two healthy individuals. The specific activity of ligase I from the crude extract was consistently lower in GM3403, a BS LCL from an Ashkenazi Jewish patient, than in normal control LCLs. By contrast, the level of ligase I activity in BSL-2KA, another BS LCL derived from a Japanese patient, was equivalent to those in normal LCLs, although GM3403 and BSL-2KA shared the feature of exceedingly high frequency of spontaneous sister-chromatid exchange. The levels of total ligase activity in crude extracts without the separation into the two forms, however, were approximately two-fold higher for the two BS LCLs than for the normal LCLs. Partial purification by chromatography on a DEAE-cellulose 23 column and a phosphocellulose column did not affect the superiority of the two BS LCLs over the normal LCLs in the specific activity of the total ligases. Nonetheless, subsequent application to an HA column again resulted in much less elevation of the specific activity of ligase I for GM3403 than for BSL-2KA and control LCLs. The levels of ligase II activity, accounting for 4–13% of total ligase activity, were similar among the LCLs examined. Irrespective of the extent of purification, essentially no difference in the heat lability of DNA ligase I was detected among the four LCLs. These findings suggest that there may exist among BS LCLs at least two types of subtle abnormality of DNA ligase I itself and/or a putative substance modulating the enzyme function.

Key words: Bloom's syndrome — DNA ligase I — Lymphoblastoid cell — Sister chromatid exchange

Bloom's syndrome (BS[5]) is a rare autosomal recessive disorder associated with an extremely increased incidence of various malignant neoplasia, growth retardation, sun-sensitive facial telangiectasia and immune disturbance.[1,2] Cells originating from BS patients exhibit an excessive number of homologous chromatid interchanges[3,4] and sister-chromatid exchanges (SCEs),[5] and also an increased frequency of spontaneous mutation[6–8] and somatic recombination.[9,10] Biochemical studies on BS cells revealed an abnormally slow rate of DNA chain growth,[11] a decreased rate of DNA fork displacement,[12] and an enhanced frequency of genetic recombination,[13] indicating that the primary defect in the disease may reside in semiconservative DNA synthesis in the S-phase.[14] However, no defect has yet been proved in the DNA polymerase functions in BS cells.[15,16]

Recently, DNA ligase I in BS lymphoblastoid cell lines (LCLs) from Ashkenazi Jewish patients and a Mennonite patient was found to exhibit reduced activity and decreased heat-stability, while the enzyme in a BS LCL from an Anglo-Saxon patient did not have such characteristics.[17–20]

In the present study, DNA ligase I activity in LCL from a Japanese BS patient was compared with that from a Jewish BS patient, and the characteristics of the enzyme activity were investigated.

MATERIALS AND METHODS

Cell lines and culture conditions GM3403, an Epstein-Barr virus (EBV)-transformed LCL derived from an Ashkenazi Jewish patient with BS (9EmSh according to the BS registry)[21] was obtained from the National Institute of General Medical Science, Camden, New Jersey. BSL-2KA, an EBV-transformed LCL derived from a Japanese patient with BS (93YoYa),[22] was established by Drs. H. Tohda and A. Oikawa, Research Institute for

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[5] The abbreviations used are: BS, Bloom's syndrome; LCL, lymphoblastoid cell line; SCE, sister chromatid exchange; HA, hydroxylapatite; DE23, DEAE-cellulose 23; P-cell, phosphocellulose.

Tuberculosis and Cancer, Tohoku University, Sendai. These BS LCLs have retained the property of a high frequency of spontaneous SCE that is a feature of the disease. NL-Ha and NL-Ch, normal control LCLs derived from healthy donors, were provided by Dr. T. Hashimoto, Hyogo College of Medicine, Nishinomiya. Cells in suspension were grown in 75-cm^2 or 150-cm^2 flasks containing RPMI-1640 medium supplemented with 4 m*M* L-glutamine, 1 m*M* α-ketoglutaric acid and 17% fetal bovine serum. All LCLs were maintained in a rapidly growing state by daily dilution to 5×10^5 cells/ml. The population doubling times for GM3403, BSL-2KA, NL-Ha and NL-Ch were 48 h, 60 h, 36 h, and 48 h, respectively. Cultures were periodically tested for mycoplasma using a DNA fluorochrome technique.[23] Approximately 1×10^8 cells were washed once with phosphate-buffered saline, pelleted by centrifugation, frozen in dry ice-acetone and stored at -80°C until use.

SCE and chromosome analysis Lymphoblastoid cells were incubated for 66 h in the dark in culture medium containing 10 μM 5-bromodeoxyuridine. Chromosome preparation and differential staining of sister chromatids were carried out according to routine methods.[24] For karyotype analysis at least 25 well-delineated metaphase spreads were prepared according to the trypsin-Giemsa banding method of Seabright.[25]

Preparation of crude extracts and partial purification Cells were thawed, homogenized in ice-cold extraction buffer [20 m*M* KPO_4 (pH 7.5), 0.5 *M* KCl, 1 m*M* dithiothreitol (DTT), 1 m*M* EDTA, 10 m*M* $NaHSO_3$, 0.5 m*M* phenylmethylsulfonyl fluoride, 2 m*M* benzamidine, 0.1 mg/ml chicken egg-white trypsin inhibitor (Boehringer Mannheim, FRG) and 0.1% Triton X-100] with a glass-Teflon homogenizer, and sonicated twice for 10 s. All the subsequent procedures were carried out at 4°C unless otherwise indicated. The homogenate was centrifuged for 1 h at 105,000*g*, and the supernatant was used as a crude extract. Essentially all of the DNA ligases I and II in culture cells can be recovered by this method of extraction.[26] For partial purification of the enzyme preparations, crude extracts were applied directly to a DEAE-cellulose 23 (DE23) column (1×2 cm), and eluted with 20 m*M* phosphate buffer [20 m*M* KPO_4 (pH 7.5), 1 m*M* DTT and 0.1 m*M* EDTA] containing 0.4 *M* KCl. Fractions of 0.4 ml were collected and those with the enzyme activity were combined and dialyzed against 1000 ml of 20 m*M* phosphate buffer for 12–16 h. The dialysates were applied to a phosphocellulose (P-cell) column (1×3 cm) and eluted stepwise with 20 m*M* phosphate buffer containing 0 *M* (5 ml) and 0.8 *M* KCl (5 ml); fractions of 0.5 ml were collected. The fractions with the ligase activity were combined and diluted with 20 m*M* phosphate buffer to adjust the concentration of KCl to 0.5 *M*.

Separation of DNA ligase I from DNA ligase II Crude extracts were first dialyzed against 1 liter of 20 m*M* phosphate buffer for 12–16 h. The dialysates were then applied to a hydroxylapatite (HA; Bio-Rad Laboratories, Richmond, CA) column (1×4 cm) equilibrated with 20 m*M* phosphate buffer, and subsequently eluted stepwise with 20 m*M* (5 ml), 0.14 *M* (5 ml) and 0.4 *M* phosphate buffer (5 ml). Fractions of 0.5 ml were collected. Partially purified preparations were also applied to an HA column and eluted with a linear gradient (10 ml) of 20–200 m*M* phosphate buffer containing 0.5 *M* KCl. Fractions of 0.7 ml were collected.

DNA ligase assay DNA ligase activity was determined essentially as described previously[27] except for the use of oligo(dT)·poly(dA) instead of calf thymus nicked DNA as a substrate. The hybrid substrate was prepared by annealing 5′-^{32}P-labeled oligo$(dT)_{12-18}$ to poly(dA) (PL Biochemicals, Uppsala, Sweden). The oligo(dT) was treated with alkaline phosphatase (Boehringer Mannheim) and then labeled with [γ-^{32}P]ATP (>5000 Ci/mmol) (Amersham, Buckinghamshire, England) by T_4 polynucleotide kinase (Takara Shuzo Co., Kyoto) in the presence of 2 m*M* KPO_4 (pH 7.5). A 10-μl aliquot of a crude extract or a chromatography fraction was incubated at 30°C for 20 min in the reaction mixture (0.2 ml) containing 75 m*M* Tris-HCl (pH 7.8), 10 m*M* $MgCl_2$, 0.2 m*M* ATP, 0.1 mg/ml bovine serum albumin, 2.5 m*M* DTT and [5′-^{32}P]oligo(dT)·poly(dA) (700–1000 cpm/pmol). Then, 0.2 ml of 0.25 mg/ml calf thymus DNA (Sigma, St. Louis, MO) as a carrier and 0.4 ml of 10% trichloroacetic acid (TCA) were added to the mixture. The precipitate was washed once with 10 m*M* HCl and completely dissolved in 100 μl of 0.1 *N* NaOH. The solution was neutralized with 50 μl of 0.3 *M* Tris-HCl (pH 7.0). Alkaline phosphatase (0.5 U) was added to the solution prior to further incubation for 30 min at 37°C. A 100-μl portion of the solution was then spotted onto a DE81 filter paper (Whatman, Maidstone, England), and the paper was washed five times with 2% K_2HPO_4. Radioactivity on the paper was measured with a liquid scintillation counter. One unit of the enzyme activity was defined as the amount converting 1 nmol of 5′-^{32}P-labeled phosphomonoesters to alkaline phosphatase-resistant diesters per min under the standard assay conditions.

Heat stability Fractions containing DNA ligase I on HA column chromatography of crude extracts and of partially purified preparations were incubated at 47.5°C and 50°C, respectively. Ten μl aliquots were subjected to DNA ligase assay after different periods of heating.

Protein content Amount of protein was determined with Bio-Rad Dye (Bio-Rad Laboratories).

RESULTS

SCEs and karyotype The two normal control LCLs, NL-Ha and NL-Ch showed basal SCE levels of 6 per cell (range 2–13), whereas GM3403 and BSL-2KA exhibited high frequencies of spontaneous SCEs per cell; 85.0± 22.2 and 93.0±13.6, respectively. NL-Ch cell line had a normal male diploid karyotype, 46,XY, and NL-Ha showed 46,XX (68%) and 47,XX,+9 (32%). Both of the BS LCLs, although diploid, displayed complex chromosome abnormalities; i.e., 46,XY,del(2),del(7),21p+ for GM3403, and 45,X,−1,−2,−6,−6,−7,−14,−17, del(12),+i(6p),+6 der t(1;2;6;7;14) for BSL-2KA.

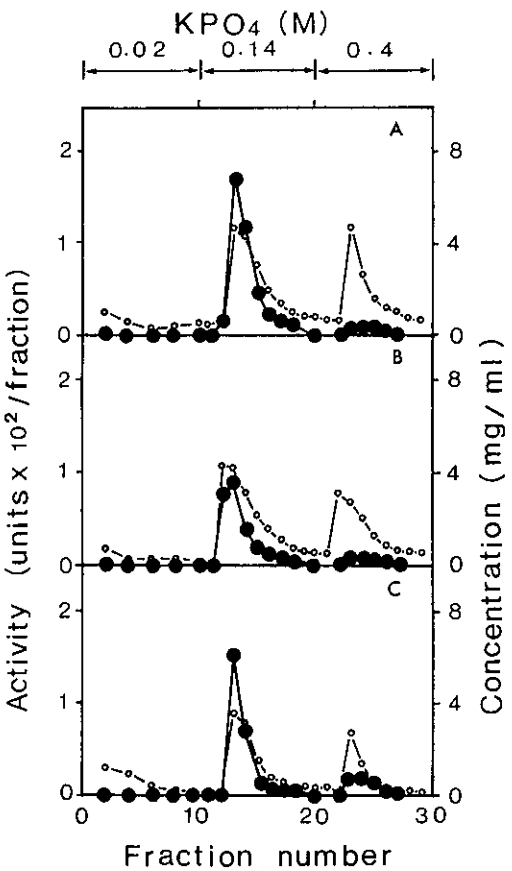

Fig. 1. Separation of DNA ligases I and II by elution on hydroxylapatite columns. Crude extracts from BS and normal LCLs were applied to HA columns and were eluted stepwise with 0.02 *M*, 0.14 *M* and 0.4 *M* phosphate buffer (0.5 ml/fraction). The protein contents of crude extracts from (A) NL-Ha (normal control), (B) GM3403 (Ashkenazi Jewish BS) and (C) BSL-2KA (Japanese BS) were 20 mg, 20 mg and 10 mg, respectively. (●), DNA ligase activity; (○), protein concentration.

DNA ligase I activity in crude extracts Following dialysis against phosphate buffer, the crude extracts from the BS and the normal control LCLs were applied to an HA column. Two peaks of the ligase activity appeared after stepwise elution (Fig. 1); i.e., a major peak corresponding to DNA ligase I eluted at 0.14 *M* phosphate buffer and the other minor peak corresponding to DNA ligase II eluted at 0.4 *M* phosphate buffer. This separation of two forms of DNA ligase on the HA column has been validated by the fact that the ligase activity associated with the major peak is completely abolished by the antibody against calf thymus DNA ligase I[26] (Teraoka, unpublished observation). The mean proportions of ligase II in repetitive chromatographic determinations for NL-Ha (n=5), NL-Ch (n=3), GM3403 (n=3) and BSL-2KA (n=3) were 4.4% (range 2.1–6.9), 13.3% (6.3–23.3), 9.8% (8.5–11.6) and 10.8% (9.9–11.8), respectively. In a representative set of experiments depicted in Fig. 1, the level of ligase I activity in GM3403 cells (2.1 mU/mg) was approximately one-half of that in NL-Ha cells (4.5 mU/mg). In contrast, BSL-2KA cells (5.4 mU/mg) had slightly higher ligase I activity than normal cells. There was no difference in the elution profile or the specific activity of ligase I between two normal controls, NL-Ha and NL-Ch (data not shown). Essentially similar results were obtained from 4 independent determinations of the enzyme activity for each cell line. Although absolute values for the specific activity of DNA ligase I varied to some extent among sets of assays, especially when different preparations of the hybrid substrate were employed for the enzyme assay, the comparative activities between GM3403 and NL-Ha and those between BSL-2KA and NL-Ha did not seem to exhibit such variations. The ratios of ligase I activity for GM3403 over NL-Ha, for BSL-2KA over NL-Ha and for NL-Ch over NL-Ha were, 0.63±0.065 (n=4), 1.12± 0.084 (n=4) and 0.89±0.093 (n=2), respectively.

Activity of total DNA ligase We assayed the total DNA ligase activity in crude extracts to see if it reflected the lower specific activity of ligase I in GM3403 compared to those in the remaining cell lines. Two independent determinations of the total ligase activity unseparated into the two forms yielded the mean values of 2.13, 3.83 and 4.29 mU/mg for NL-Ha, GM3403 and BSL-2KA, respectively. Thus, the total ligase activities of the two BS LCLs were almost two-fold higher than that of NL-Ha, notwithstanding the clear distinction between the two BS LCLs regarding the ligase I activity eluted from HA columns.

To get some insight into the cause of the discordance of total ligase activity and the DNA ligase I activity in GM3403 cells, we compared first the effect of partial purification on the specific activity of the total ligase among NL-Ha, BSL-2KA and GM3403. Crude extracts

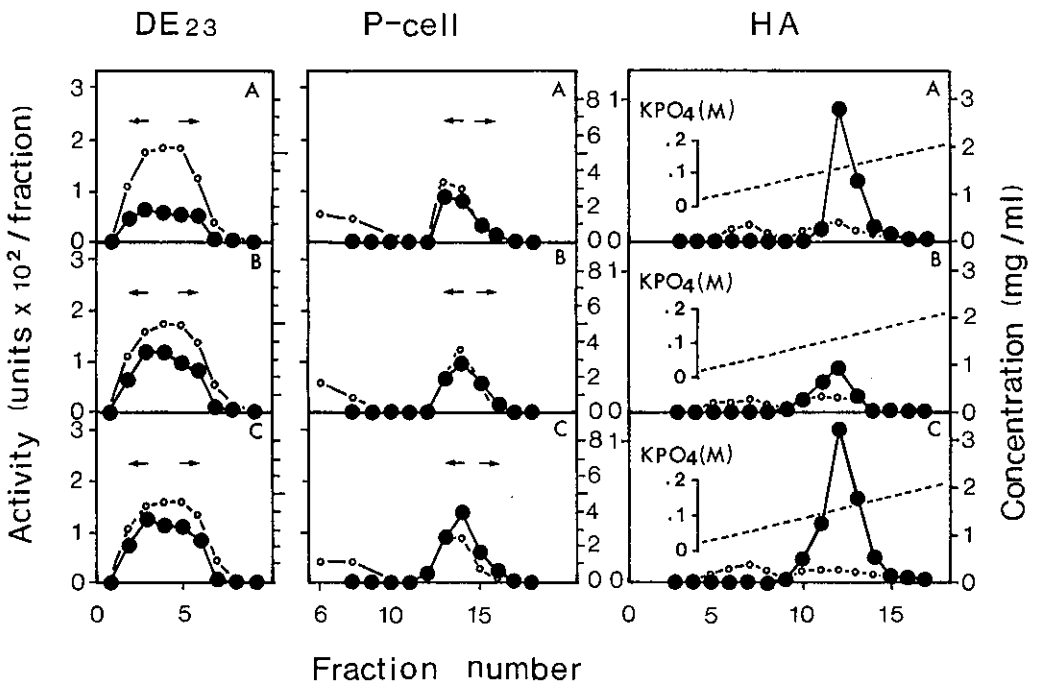

Fig. 2. Partial purification and subsequent hydroxylapatite column chromatography of DNA ligase from BS LCLs and normal LCLs. Crude extracts were passed through a DEAE-cellulose column (0.4 ml/fraction) (left panel, DE23). The peak fractions (fraction No. 2–6) were combined, dialyzed, applied to a phosphocellulose column and eluted stepwise with phosphate buffer without (fraction No. 1–10) and with 0.8 *M* KCl (fraction No. 11–20) (0.5 ml/fraction) (middle panel, P-cell). The fractions with the activity (fraction No. 13–16) were combined, diluted to 0.5 *M* KCl, applied to a hydroxylapatite column and eluted with a linear gradient of 20–200 m*M* phosphate buffer (0.7 ml/fraction) (right panel, HA). (A) NL-Ha (normal control); (B) GM3403 (Ashkenazi Jewish BS); (C) BSL-2KA (Japanese BS). Closed circles, DNA ligase activity; open circles, protein concentration. Fractions marked by arrowheads denote peak fractions pooled for further steps.

from NL-Ha (24.6 mU, 13.6 mg protein), GM3403 (48.4 mU, 13.3 mg) and BSL-2KA (50.0 mU, 14.0 mg) were loaded on DE23 columns, and total ligase activities (total protein) recovered were 22.9 mU (9.0 mg), 45.5 mU (8.6 mg) and 45.5 mU (8.2 mg), respectively (the left panel, Fig. 2). When each preparation was subjected to P-cell column chromatography, the eluates contained 22.9 mU (3.8 mg), 23.8 mU (3.2 mg) and 31.6 mU (3.5 mg), respectively (middle panel, Fig. 2). Thus, the partial purification by chromatography on DE23 and P-cell columns, which resulted in a several-fold increase in the specific activity of the total DNA ligase, did not affect the relative abundance of the enzyme activity in the two BS LCLs (Table I). The P-cell eluates were applied to HA columns, and the peak fractions corresponding to DNA ligase I for NL-Ha, GM3403 and BSL-2KA were found to contain 14.9 mU (0.91 mg), 9.4 mU (1.06 mg) and 23.7 mU (1.10 mg), respectively (right panel, Fig. 2). Reduced specific activity of DNA ligase I in GM3403 became evident at this step of purification, as the overall recoveries of ligase activity for NL-Ha, GM3403 and BSL-2KA were 60%, 19% and 47%, respectively. Although the ligase I activity of BSL-2KA remained higher than that of NL-Ha, the overall degree of purification of

Table I. Partial Purification of DNA Ligase I from Human Lymphoblastoid Cells[a]

Purification step	Specific activity of DNA ligase[b]		
	NL-Ha[c]	GM3403[d]	BSL-2KA[e]
I. Crude extract	1.80 (1.0)	3.63 (1.0)	3.57 (1.0)
II. DE23	2.53 (1.4)	5.31 (1.5)	5.52 (1.6)
III. P-cell	5.90 (3.3)	7.46 (2.1)	9.02 (2.5)
IV. HA[f]	16.61 (9.2)	8.91 (2.5)	21.63 (6.1)

a) Summary of the purification of DNA ligase I, whose chromatographic properties are shown in Fig. 2.
b) Values are in mU/mg (fold increase).
c) LCL from a normal control.
d) BS LCL from a Ashkenazi patient.
e) BS LCL from a Japanese patient.
f) Values are for DNA ligase I only.

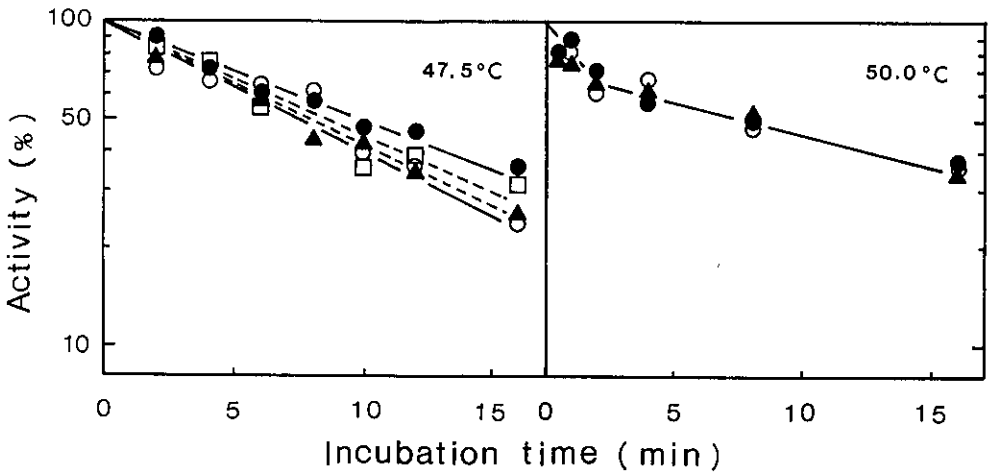

Fig. 3. Heat stability of DNA ligase I in BS and normal LCLs. The peak fractions containing DNA ligase I from HA column chromatography were incubated at 47.5°C for crude extract as depicted in Fig. 1 (left), or at 50.0°C for partially purified preparation as shown in the right panel of Fig. 2 (right). Lines were fitted by eye. (□) NL-Ha; (○) NL-Ch; (▲) GM3403; (●) BSL-2KA.

ligase I for BSL-2KA was lower than that for NL-Ha (Table I).

Heat stability of DNA ligase I To examine whether BSL-2KA LCL and GM3403 LCL differ in the heat stability of DNA ligase I, the top fractions from HA column chromatography were incubated at 47.5°C for crude extracts and at 50.0°C for partially purified preparations. DNA ligase I in partially purified preparations from LCLs was slightly more resistant to heat than that in the crude extracts. No significant difference was noted in the heat stability among the four LCLs examined (Fig. 3).

DISCUSSION

In the present study, we demonstrated that the activity level of DNA ligase I in the crude extracts after separation on HA columns was lower in GM3403 than in BSL-2KA and control LCLs (Fig. 1) and that the total DNA ligase activity was 2-fold higher in the BS LCLs than in normal LCLs (Table I).

The former observation appears to be in line with the previous reports by Willis and Lindahl[17,20] that the size-fractionation of the cell extracts by liquid chromatography on an FPLC Superose-12 column yielded a reduced amount of DNA ligase I activity for GM3403. The specific activities of the ligase I in extracts from the normal control cell lines appeared to be much lower in their FPLC analyses[17] than in our HA chromatographic analyses. Furthermore, the ratio of ligase I to ligase II separated by FPLC size-fractionation was 1.5–2.0 in extracts from the control lines in the previous reports,[17,20] while the I/II ratio was much higher on the HA chromatography in the present study (Fig. 1), as reported with DNA ligases I and II from calf thymus[28] and from rat liver epithelial cells.[29] Willis *et al.* treated crude cell extracts with 0.5% Polymin P in the presence of 0.1 *M* NaCl to remove coexisting nucleic acids prior to size-fractionation and DNA ligase assay.[17,20] We suspect that this treatment with Polymin P resulted in the preferential precipitation of DNA ligase I bound to the nucleic acids,[30] leading to the lower recovery of the ligase I in the supernatant to be analyzed.[31]

The amount of DNA ligase I has been found to increase up to 15-fold in dividing cells,[32] suggesting a role of this enzyme in semiconservative DNA replication. BS LCLs employed in this study were relatively slow-growing compared to the normal counterparts, but GM3403 displayed rather a shorter population doubling time (48 h) than BSL-2KA (60 h). All of the four LCLs examined including GM3403 showed diploidy or near-diploidy. Therefore, neither a differential proportion of cells in growth fraction nor a gene dosage effect is likely to account for the difference in the ligase I activity between the two BS LCLs.

It was unexpected that the total activity of unseparated ligases in crude extracts was two-fold higher for both GM3403 and BSL-2KA than for control LCLs, despite the distinction between GM3403 and BSL-2KA in the ligase I activity eluted from HA columns. Since the ratio of ligase I to II after chromatographies on HA columns (this study) and also FPLC Superose 12 columns (Kurihara and Teraoka, unpublished observation) was similar between BS and control LCLs, the higher activity

of total DNA ligase in BS cells does not seem to result from an increase in ligase II activity but from an enhancement of ligase I activity. Mezzina *et al.* also reported that the total ligase activity in crude extracts from four BS cell strains and cell lines including GM3403 was significantly higher than that in control cells.[31] They postulated that this increase in the total ligase activity was to counteract the higher levels of the deoxyribonuclease activity in BS cells.

The superiority of the two BS LCLs over the normal LCLs in the specific activity of the total ligase persisted even after cellular DNA as well as proteins with no affinity for phosphate residues were removed from crude extracts by chromatographies on DE23 and P-cell columns (Fig. 2). A critical step leading to the decrease in DNA ligase I activity of GM3403 is obviously the HA column chromatography (Table I and Fig. 2, right panel). The increase in the specific activity of ligase I for BSL-2KA at this step of purification was also somewhat smaller than for NL-Ha (Table I). It remains to be established why DNA ligase I activity in GM3403 was reduced on elution from HA columns. One possibility would be that the altered ligase I in GM3403 is trapped or inactivated by the HA column. Alternatively, a putative substance modulating the DNA ligase reaction could be selectively lost or retained only for GM3403 on HA column chromatography due to its altered properties in GM3403. The latter interpretation is consistent with the recent discoveries of an activator[33] and an inhibitor[34] for the ligase I reaction. Hence, we suppose that an unidentified dysfunction of DNA ligase I activity of BS LCLs incurs a compensatory alteration in gene expression of the enzyme itself or some stimulatory factor(s) for the enzyme, leading to the apparent increase in total ligase level.

An alteration in the heat-lability of a protein can be indicative of a mutation in the structural gene. In contrast with the previous report by Willis *et al.*,[17,20] we could not detect any difference in the heat sensitivity of DNA ligase I, whether partially purified or not, between BS LCLs and control LCLs (Fig. 3). The DNA ligase I in partially purified preparations was significantly more resistant to heat than that in crude extracts, presumably reflecting the removal of some substance which destabilizes the ligase I. Mezzina *et al.* also failed to observe the heat-lability of DNA ligase from GM3403.[31] Since ligase I is essential for DNA replication, BS cells should bear only leaky mutation at the gene coding for ligase I itself or a putative auxiliary protein. The deviation as to the properties of DNA ligase I activity in BS LCLs may, therefore, be too subtle to bring about the conspicuous heat lability. The molecular cloning of the structural gene for the enzyme or for the regulatory protein(s) is mandatory for fuller understanding of the minimal change in BS ligase I function.

So far no complementation of the high SCE characteristic of BS cells has been observed with hybrid lines derived from patients of Ashkenazi Jewish, French-Canadian, Mennonite, or Japanese extraction,[35] implying that BS in both the Japanese and the non-Japanese population can be ascribed to mutation at a single locus. A different class of mutation or a mutation at a different site within the same locus could result in some difference in the mutant phenotype. Based on the chromatographic properties of DNA ligase I on an HA column (Fig. 1), BS LCLs can be classified into at least two types. In view of this distinction, it is noteworthy that the clinical phenotype in Japanese patients with BS differs from most typical cases, including Ashkenazi Jewish, in that dolichocephaly is less constant, the facial skin lesion is less prominent, and life-threatening infections are rare.[22] To date, four BS LCLs from Ashkenazi patients and one BS LCL from a Mennonite patient have been characterized as having a reduced activity and decreased heat-stability of DNA ligase I.[17-20] One LCL derived from an Anglo-Saxon patient with BS and also a BS fibroblast strain derived from another Japanese patient, 86NoKi, lacked these characteristics.[20,36] Further examinations on additional BS LCLs from Japanese patients are under way to determine if BS in Japanese resemble that in Anglo-Saxons in this regard.

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