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Genetics

Structural alterations of DNA ligase I in Bloom syndrome

(chromosome instability/DNA-replication defect/cancer-prone inherited disease)

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ABSTRACT **Cell lines derived from seven patients with Bloom syndrome all contain a DNA ligase I with unusual properties. Six lines were shown to have a reduced level of this enzyme activity and the residual enzyme was anomalously heat-labile. The seventh line contained a dimeric rather than monomeric form of ligase I. Several cell lines representative of other inherited human syndromes have apparently normal DNA ligases. The data indicate that Bloom syndrome is due to a defect in the structure of DNA ligase I caused by a "leaky" point mutation occurring at one of at least two alternative sites.**

The rare syndrome first described by Bloom in 1954 as a "congenital telangiectatic erythema resembling lupus erythematosus in dwarfs" (1) is associated with a greatly increased incidence of cancer. Thus, in a recent survey of this recessively inherited disease, 28 malignant neoplasms of several different types were detected in 103 young patients (2). Most cases of Bloom syndrome (BS) have been found among Ashkenazim, and it has been estimated that about 1% of this population represent BS heterozygotes (3). However, the disorder has also been reported in non-Jewish individuals, including American blacks (4) and Japanese (5).

Cells from BS patients exhibit frequent chromosome rearrangements [in particular, symmetrical quadriradials indicative of exchanges between homologous chromatids at homologous sites (6, 7)], and a 15- to 20-fold increased level of spontaneous sister-chromatid exchange (SCE) is regarded as a hallmark of the disease (8). In addition, BS cells show a 5- to 10-fold elevated spontaneous mutation rate (9). Ten years ago, Giannelli *et al.* (10) found that replicative intermediates of DNA exhibit delayed maturation into a very high-molecular weight form in BS cells and suggested that the fundamental defect involves a step in DNA replication. A slight decrease in the rate of DNA fork displacement during replication has also been observed for BS cells (11, 12). Moreover, such cells show slightly increased sensitivity to ultraviolet and near-ultraviolet light accompanied by altered unscheduled DNA synthesis and increased chain breakage compared to control cells, indicative of an abnormality in a postincision step of DNA excision–repair (13, 14).

We have shown previously (15) that the level of activity of the major DNA ligase in proliferating human cells, ligase I, is reduced in the BS-derived lymphoblastoid cell line GM3403. Furthermore, the ligase I in these cells is anomalously heat-labile, indicating a mutation in the structural gene for DNA ligase I in this line (15). Preliminary observations on altered aggregation properties of ligase I from other BS cell lines have also been made (15, 16). In the present work, we extend our molecular analysis to six additional BS cell lines and show that two clearly distinguishable types of ligase I defects occur in patients with this clinical syndrome.

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MATERIALS AND METHODS

Cell Lines and Cell Culture. Five BS cell lines were derived from different Ashkenazi BS patients. The simian virus 40-transformed fibroblast line GM8505 and the lymphoid cell line GM3403 were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). The lymphoid lines W67-4, D86-1-2, and AA87-5-1 were established by immortalization with Epstein–Barr virus as described (17). The lymphoid line AA87-4 was from a parent of the AA87-5-1 donor. The latter four lines were made available by E. E. Henderson (Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia). Two lymphoid lines of non-Ashkenazi origin were established from Canadian children having both BS and Wilms tumor; 1004 was from a Mennonite and 1032 was from an Anglo-Saxon patient. More detailed information on the origin of these two lines will be presented elsewhere (30). Lines representative of other human inherited diseases were from the Human Genetic Mutant Cell Repository, as follows: AG3829, Werner syndrome; GM2449, xeroderma pigmentosum variant; GM1712, Cockayne syndrome; GM1953, control from a healthy individual. The lymphoid cell line PS from a Friedreich ataxia patient was obtained from Susan Chamberlain (St. Mary's Hospital, London). Cells were grown at 37°C in media supplemented with 15% fetal bovine serum, fibroblasts in Dulbecco's modified Eagle's medium and suspension cultures of lymphoid cells in RPMI 1640. SCE frequency was determined according to Perry and Wolff (18).

Enzyme Determinations. Crude cell extracts were treated with Polymin P (BDH) to remove nucleic acids and size-fractionated by FPLC (Pharmacia) prior to assay for DNA ligase activities as described (15). The salt concentration was maintained at 0.05–0.2 M NaCl throughout these procedures. Sucrose gradient centrifugation of cell extracts was performed according to Martin and Ames (19). DNA (guanine-O^6)-methyltransferase was assayed as described (20).

RESULTS

SCE. Several control lymphoid cell lines, including line GM1953 from a healthy individual, had basal SCE frequencies of 4–8 per cell. As expected, the BS lines GM3403, GM8505, W67-4, 1004, and 1032 exhibited high numbers of SCE, 60–80 per cell. In contrast, the two BS lines D86-1-2 and AA87-5-1 showed a SCE frequency of only 12–18 per cell and were therefore different from the majority of the BS lines as well as from the control cell lines (E. E. Henderson, personal communication). Thus, cell lines derived from BS patients fall into two distinct categories with respect to the magnitude of the increase in spontaneous SCE frequency.

Enzyme Deficiency in BS Lines. DNA ligases I and II in cell extracts were separated by FPLC size fractionation. In control cells, enzyme assays revealed a major peak of the

Abbreviations: BS, Bloom syndrome; SCE, sister-chromatid exchange.

200-kDa ligase I, followed by a smaller peak of the 80-kDa ligase II. The latter enzyme was also identified by its unique ability to join nicks in the DNA strand of a DNA·RNA hybrid (21). The ratio of ligase I to ligase II activity was 1.5–2.0 in extracts from the lines GM1953 (healthy control), GM1712 (Cockayne syndrome), AG3829 (Werner syndrome), GM2449 (xeroderma pigmentosum variant), and PS (Friedreich ataxia) (Figs. 1 and 2). There was no indication of a DNA ligase deficiency in any of these diseases. In contrast, the level of ligase I in the BS line W67-4 was one-third of that found in normal cells (Fig. 1*a*) and was indistinguishable from that of the BS line GM3403 investigated previously (15). Similar results were also obtained with a simian virus 40-transformed fibroblast line (GM8505) of BS origin (Fig. 1*b*). These three lines were derived from Ashkenazi patients, but a BS line from a Mennonite child (line 1004) also yielded very similar results (Fig. 1*c*). These data show that DNA ligase I levels are markedly reduced in several representative BS cell lines.

Enzyme Levels in BS Lines with Low SCE Frequency and in a Heterozygote Line. Six different BS lymphoid cell lines were available for study, four with the expected high level of SCE and two (D86-1-2 and AA87-5-1) with a slightly increased level. The latter, as well as lines with high SCE, showed anomalously low levels of DNA ligase I activity in cell extracts (Fig. 2). Thus, the reduction in ligase I activity was independent of the size of the increase in SCE frequency. The single available parental heterozygote cell line of BS, AA87-4, although containing a higher level of ligase I than the BS cells (Fig. 2*b*), had an activity that was apparently lower than any of the lines representative of healthy individuals or other syndromes, as anticipated. However, due to experimental variations this heterozygote line cannot be distinguished with certainty from some members of the control group. That is, the apparent difference between the experiments with the xeroderma pigmentosum variant line (Fig. 1*c*) and the Friedreich ataxia line (Fig. 2*a*) is as large as the difference between the latter and the AA87-4 line.

Second Type of Ligase I Alteration. The Anglo-Saxon BS line, 1032, was unlike the other BS lines in that no DNA ligase I monomer was detected after size fractionation. Instead, a peak of DNA ligase activity was eluted early on FPLC fractionation, although clearly separated from the void volume (Fig. 3*a*). When extracts of line 1032 were made 1 M with respect to NaCl, one hour before chromatography, the ligase activity was converted to a form that was eluted at the expected position of DNA ligase I. The data strongly indicate that, in line 1032 cells, ligase I is present as a dimer (or, less likely, as a monomer tightly bound to another protein of the same size) that can be dissociated by high salt treatment. A slower, partial conversion to monomer form occurred in the presence of 0.2–0.5 M NaCl. These observations were confirmed by sucrose gradient centrifugation experiments. In comparison with reference proteins, DNA ligase I from control cells had a sedimentation coefficient of 8–9 S (data not shown), and the same value was obtained for the residual ligase I activity in the Ashkenazi BS line W67-4 (Fig. 3*b*); the 1032 material showed a small peak of ligase I at this position, but most of the activity appeared as a distinct peak at 13 S. By combining the Stokes radius data obtained by gel filtration and the sedimentation coefficients in the Svedberg equation (22), molecular weights of approximately 200,000 and 400,000, respectively, were estimated for the two forms of ligase I.

Heat Lability of Ligase I in BS. Peak fractions of DNA ligase I from FPLC experiments were incubated at 50°C, and aliquots were removed at different times for ligase assays. The enzyme activity from all the non-BS lines decreased with apparent first-order kinetics and showed 50% inactivation in 6 min. In contrast, the ligase I from the BS lines W67-4, GM3403, GM8505, 1004, D86-1-2, and AA87-5-1 was clearly more heat-labile, being 50% inactivated in 3 min (Fig. 4 and data not shown). These results confirm that the increased

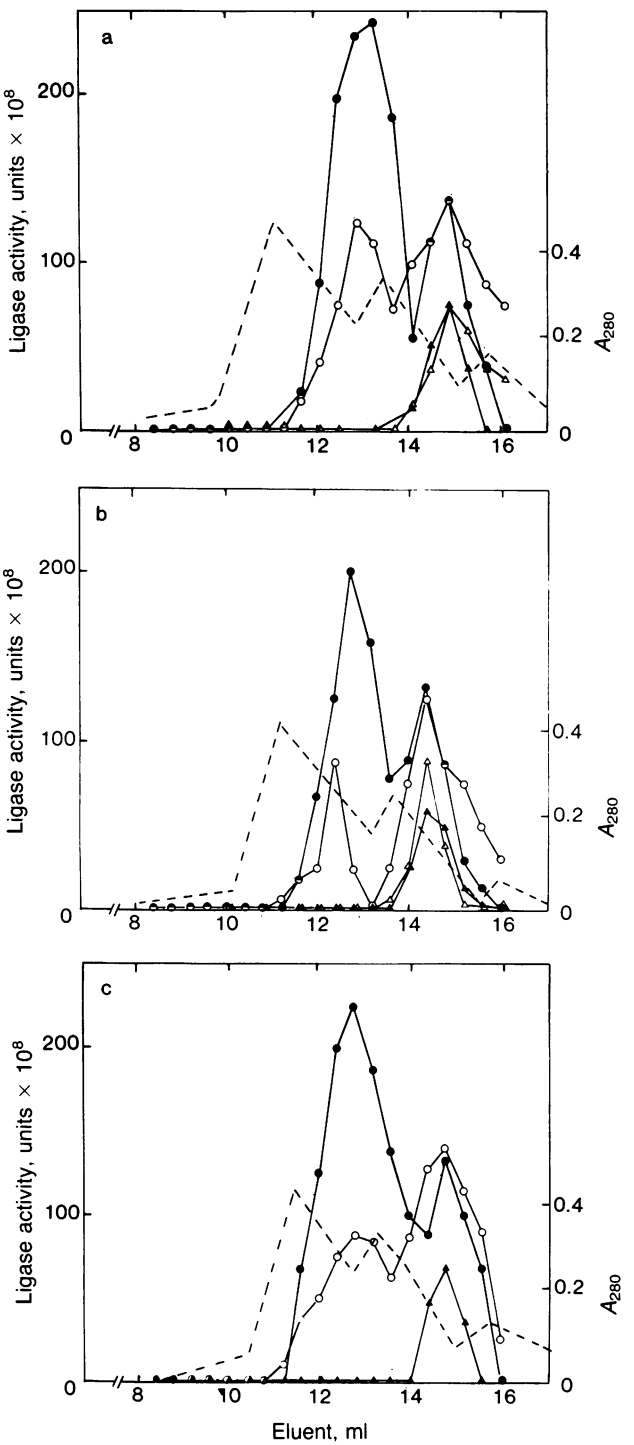

FIG. 1. Size fractionation of DNA ligase activities in representative BS cell lines. FPLC Superose-12 column profiles of Polymin P-treated cell extracts are shown. (*a*) Lymphoid cell lines W67-4 (Ashkenazi BS) and AG3829 (Werner syndrome). (*b*) Simian virus 40-transformed fibroblast line GM8505 (Ashkenazi BS) and HeLa cells. (*c*) Lymphoid cell lines 1004 (Mennonite BS) and GM2449 (xeroderma pigmentosum variant). Circles show results with the standard DNA ligase assay (15), and triangles show results with the DNA·RNA hybrid assay specific for DNA ligase II. (Line 1004 was not tested with the latter assay.) Open symbols depict the results with BS lines, and closed symbols, other cell lines. Broken line represents A_{280} of the BS material; the A_{280} profiles of the controls were similar.

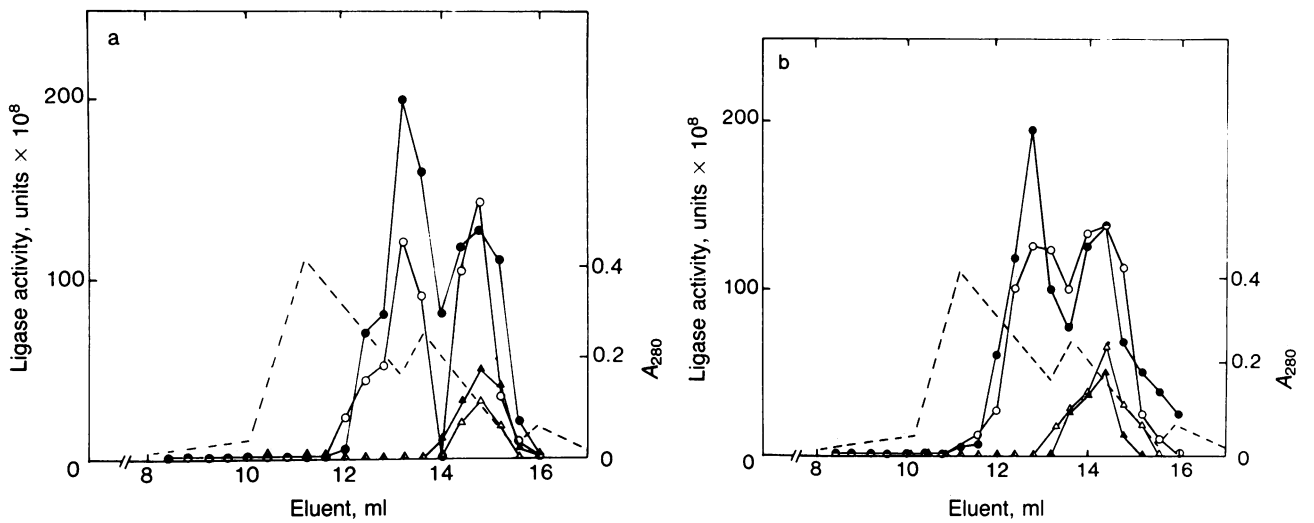

FIG. 2. DNA ligase activities in BS lymphoid cell lines with low SCE and in a heterozygote line. Chromatographic conditions and symbols are as in Fig. 1. (*a*) Lines D86-1-2 (Ashkenazi BS) and PS (Friedreich ataxia). (*b*) Lines AA87-5-1 (Ashkenazi BS) and AA87-4 (heterozygote; parent of patient from which AA87-5-1 was derived).

lability reported previously for the GM3403 line (15) is characteristic of ligase I from a number of BS lines. The dimeric form of ligase I from the BS line 1032 was not heat-sensitive, however, and was indistinguishable from the control material in this regard (Fig. 4). Moreover, the stability of the ligase I activity from the heterozygote AA87-4 could not be distinguished from that of control cells, presumably because most of the activity in this line was due to the normal enzyme.

Several other properties of the altered forms of DNA ligase I from lines GM3403 and 1032 were investigated. They did not differ markedly from ligase I from control GM1953 cells with regard to pH dependence, K_m for the ATP cofactor, ability to use Mn^{2+} instead of Mg^{2+} as cofactor, or inhibition with increasing concentrations of NaCl, although the enzyme from GM3403 cells was slightly more sensitive in the latter regard (70% inhibition by inclusion of 0.1 M NaCl in the standard reaction mixture vs. 50% inhibition of ligase I from 1032 and GM1953 cells).

Mex Phenotypes. Human lymphoid cell lines may be of either Mex^+ or Mex^- phenotype, the latter being anomalously sensitive to alkylating agents (23). Mex^- cells do not contain an active DNA (guanine-O^6)-methyltransferase (20) and have been reported to show delayed joining of strand interruptions in DNA (24). However, from methyltransferase assays no correlation between the Mex phenotype and levels of ligase activity was observed; i.e., the BS lines GM8505 and D86-1-2 were Mex^+, W67-4, GM3403, and 1032 were Mex^-, and 1004 appeared intermediate (data not shown).

DISCUSSION

There appears to be a consistent correlation between BS and a structural defect of DNA ligase I. Cell lines derived from seven different BS patients were investigated, and all of them contain a DNA ligase I with altered properties, whereas ligase II is normal. These data both confirm and extend our previous observation (15) that an anomalously heat-labile DNA ligase I activity was present in the BS lymphoid cell line GM3403. In contrast, no unusual properties of DNA ligases were detected in 10 different human cell lines derived from normal individuals or from patients with inherited diseases other than BS. Thus, no DNA ligase defect was observed in ataxia-telangiectasia, Fanconi anemia, Werner syndrome, xeroderma pigmentosum (including the variant complementation group), Friedreich ataxia, and Cockayne syndrome (ref. 15 and this work).

Two different types of structural alteration in DNA ligase I from BS cells were characterized. The five cases of BS in Ashkenazim all showed the same type of molecular defect, exhibiting markedly reduced activity and decreased heat stability of the enzyme under our assay conditions. These observations are consistent with the hypothesis that most Jewish individuals with BS are descendants of a single founder living in Poland centuries ago (2). A single Canadian Mennonite BS case with a history of parental consanguinity was also investigated and found to exhibit a ligase defect indistinguishable from that seen in the Ashkenazi material. In view of the relative isolation of the Mennonite community, it seems likely that this case represents an independently derived but similar mutation. In contrast, the DNA ligase I alteration seen in the 1032 cell line derived from an Anglo-Saxon BS patient is clearly different, because at low or moderate ionic strength the enzyme was present as a 400-kDa dimer rather than a 200-kDa monomer, and no heat lability was observed. Preliminary experiments with fibroblasts from a Japanese BS patient (15) yielded results for ligase I indistinguishable from those documented in more detail here with the 1032 line. These data suggest that a different mutation is present in the gene for ligase I in the latter two cases, again resulting in the synthesis of a defective form of the enzyme. We refer to these different molecular alterations as ligase defects I-1 (heat-labile enzyme) and I-2 (dimeric enzyme). It is not surprising that a DNA ligase I of reduced activity can be due to alternative mutations; by comparison, malfunctioning hemoglobin variants are known to result from several different point mutations (25). There does not seem to be any obvious distinction in the clinical symptoms of BS between the two types observed here. Further, no genetic evidence has been obtained for heterogeneity in BS. Fusion of type I-1 and type I-2 cells has demonstrated noncomplementation of the increased SCE frequency (30), whereas fusion of BS cells with normal cells results in suppression of the elevated SCE (26).

Recently, Chan *et al.* (16) reported that 15–40% of the DNA ligase I activity (termed ligase Ia) appeared close to the void volume rather than at the position of the ligase I monomer following high-salt extraction of lymphoid cells and gel filtration. The amount of this large form of the enzyme, but not the monomer, seemed reduced in extracts from BS cells and showed an apparent 20% reduction in molecular

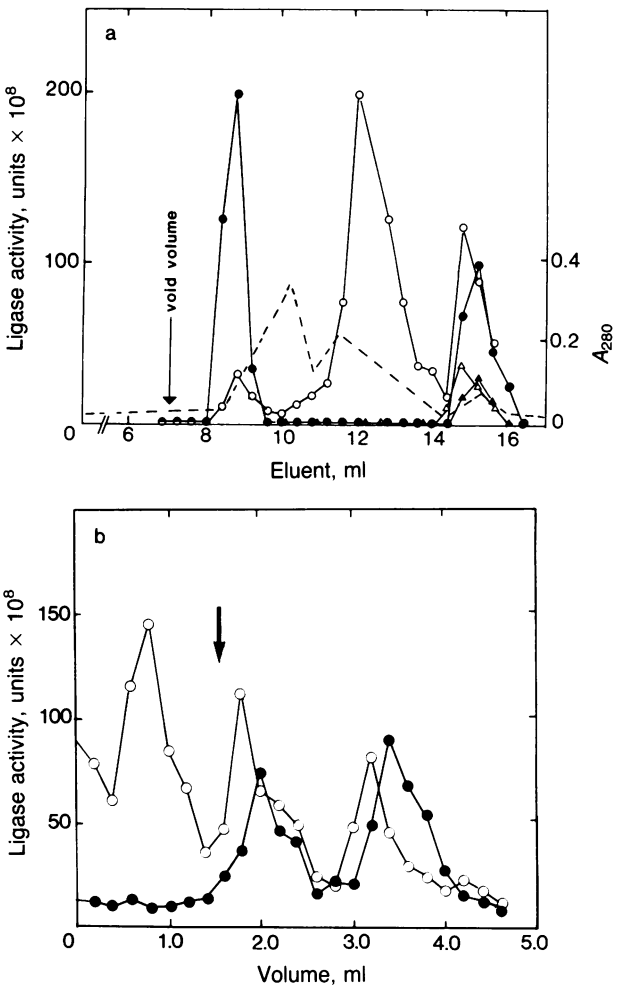

FIG. 3. DNA ligase activities in the Anglo-Saxon BS line 1032, containing a dimeric form of ligase I. (*a*) FPLC profiles as in Fig. 1 for closed symbols. The open symbols show the same experiment, but with incubation of the cell extract in 1 M NaCl prior to chromatography. (*b*) Sucrose gradient centrifugation. Cell extracts from the BS lines 1032 (○) and W67-4 (●), 0.2 ml each, were layered on 5-ml sucrose gradients (5–20%) containing 200 mM NaCl, 50 mM Tris·HCl (pH 7.5), 10 mM 2-mercaptoethanol, and 1 mM EDTA. Gradients were centrifuged at 40,000 rpm in a Beckman 50.1 rotor for 10 hr at 4°C. Fractions were collected from the bottom of the tube and assayed for ligase activity. The molecular weight standards used were catalase, which was also added to the extracts as an internal marker (indicated by arrow), apoferritin, alcohol dehydrogenase, and alkaline phosphatase. Direction of sedimentation is towards the left.

weight compared with ligase Ia from control cells. These observations are not easy to reconcile with our findings; in high-salt buffers we do not detect any differences in aggregation state between ligase I from BS and control material, nor do we find a difference in molecular weight between the BS and control enzymes.

Several BS patients exhibit two populations of circulating lymphoid cells, a major one with high SCE and a minor one with low SCE (8, 27); fibroblasts from such individuals all seem to show high SCE. Since spontaneous revertants of a specific single-site mutation would be expected to be quite rare, the low SCE population is unlikely to represent back-mutations, in spite of the increased mutation frequency in BS. A comparatively low SCE frequency is present in two of the six BS lymphoid cell lines investigated, that is, a 2- to 3-fold, rather than the more usual 15- to 20-fold, increase in SCE compared to controls. Interestingly, the DNA ligase I defect was retained in such cells, and they could not be distinguished from BS lines showing high SCE in our biochemical experiments. It seems likely that a

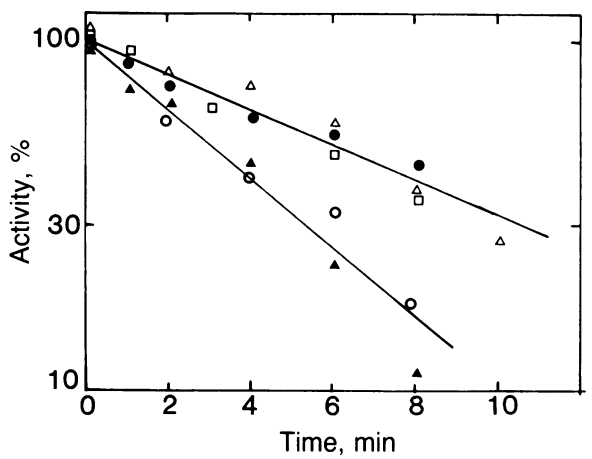

FIG. 4. Heat lability of DNA ligase I in BS. Ligase I peak fractions from FPLC were incubated at 50°C, and aliquots were removed at various times and assayed as described (15). The symbols indicate material from Ashkenazi BS line W67-4 (○), Mennonite BS line 1004 (▲), Anglo-Saxon BS line 1032 (□), BS heterozygote AA87-4 (△), and control line GM1953 (●).

compensatory change has occurred in the DNA-replication machinery of these cells, presumably involving overproduction or alteration of another replication factor. A change of this type may also account for the observation (28) that some BS cell lines exhibit high SCE as well as a high level of chromosome breakage, whereas other lines retain the high SCE but no longer show chromosome instability.

Our data support a model in which BS is due to a missense mutation in the structural gene for DNA ligase I, resulting in a malfunctioning variant of this essential enzyme. The increased rates of mutagenesis, chromosome breakage, and somatic recombination caused by this defect could lead to a general predisposition to cancer (29).

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