Mammalian DNA ligases: Characterization of their Biochemical properties and a defect in Bloom's syndrome

by

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Abstract

Mammalian cells contain two DNA ligases, namely DNA ligase I and II. Based on their differing substrate specificities, I have developed specific assays for each of these two enzymes. Both enzymes can form phosphodiester bonds in a homopolymer, poly(dA)·oligo(dT) substrate, but DNA ligase II is also capable of joining strand breaks in a hybrid poly(rA)·oligo(dT) substrate. DNA ligase I and II can seal strand breaks in 'nicked' plasmid DNA, but DNA ligase I is the only enzyme which is able to join DNA which has been cut to give blunt ends.

The assays developed herein have been used to distinguish between the two DNA ligase activities in cell lines derived from several inheritable cancer prone syndromes. With the exception of Bloom's syndrome cells, all of the cell lines studied have normal levels of DNA ligase I and II, with a ratio of DNA ligase I:DNA ligase II of approximately 1.6:1. However, in cell lines independently derived from six Bloom's syndrome patients of Ashkenazi Jewish origin, the activity of DNA ligase I was reduced by approximately 70% such that the ratio of DNA ligase I:DNA ligase II was 0.5:1. Furthermore, the DNA ligase I from these Bloom's syndrome cell lines was found to be twice as heat labile as the enzyme from non-Bloom's syndrome cells.

In three cell lines derived from Bloom's syndrome patients of non-Jewish origin, a different type of defect has been found. This defect manifests in an enzyme that dimerizes to give a large proportion of the active DNA ligase I with a molecular weight of ~400. These dimers are present under physiological salt concentrations, but can be dissociated by high ionic strength. The DNA ligase I from these cells is not heat labile.

These data indicate that Bloom's syndrome cells produce a defective DNA ligase I. The two types of structural defects observed may result from the expression of two different point mutations.

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Chapter 1

Introduction

Introduction

1.0 For proliferation and maintenance of living cells, pathways for growth, DNA replication, DNA repair and cell division must be carefully co-ordinated. Certain enzyme systems are of fundamental importance to these processes, as reflected in the conservation of their activities throughout the animal and plant kingdoms. One such activity, that of DNA ligase, is universally necessary for the replication and repair of DNA. This thesis presents a study of mammalian DNA ligases and the relationship of DNA ligase I to Bloom's syndrome, an inheritable cancer prone disease.

1.1 Biochemical Characteristics of Mammalian DNA Ligases

The presence of a DNA ligase activity in eukaryotic cells was first reported by Lindahl & Edelman (1968). This enzyme was observed to be nuclear in origin and similar to the bacteriophage T4 enzyme in that it had an absolute requirement for adenosine triphosphate (ATP). A second nuclear DNA ligase activity was subsequently discovered by Soderhall & Lindahl (1973) which was termed DNA ligase II.

Purification and Characterization of these two proteins indicated a number of differences in their properties. Hence, DNA ligase I had a molecular weight of 180000 whereas DNA ligase II is a smaller protein of

80kDa. Both enzymes require ATP for activity, although DNA ligase I has a greater affinity for ATP with a Km of $1x10^{-6}$ whereas that for ligase II is $1x10^{-4}$. The two enzymes exhibit the same pH optimum (pH7.8), however, DNA ligase I is active over a wider pH range than DNA ligase II (Soderhall & Lindahl, 1976). DNA ligase II is more heat labile with a half life of 5 min at 42°C, in contrast to ligase I which has a half life of 5 min at 52°C (Soderhall & Lindahl, 1973a). Polyclonal antiserum directed against 1000 fold purified DNA ligase I does not cross react with DNA ligase II indicating that these enzymes may be serologically distinct (Soderhall & Lindahl, 1975).

The existence of two different DNA ligases has been the subject of controversy, probably because DNA ligase I is very susceptible to proteolysis, which gives rise to a range of active peptides, many of them having a similar size to DNA ligase II. The size heterogeneity of ligase I has been commented on by Pedrali Noy *et al.* (1973) who found that upon ageing of cell extracts a conversion of a 190kDa form of the enzyme to a 95kDa form occurred.

Teraoka and Tsukada (1982) purified an active 130kDa fragment of DNA ligase I, and suggested that DNA ligase II was a product of partial proteolysis of DNA ligase I. Evidence to support this contention came from experiments where the addition of the protease inhibitor, phenylmethylsuphonate fluoride, to nuclear extracts

subsequently inhibited the production of "ligase II". A range of fragment sizes for DNA ligase I has also been detected on 'activity gels' and it has been postulated that programmed proteolytic cleavage of the enzyme occurs *in vivo* to regulate DNA replication processes at initiation and elongation stages (Mezzina *et al.*, 1984).

1.2 Mechanisms of Action of DNA Ligases

DNA ligase activities were initially characterized from both T_4 infected and uninfected *E. coli* (Weiss & Richardson, 1967; Becker *et al.*, 1967; Gellert, 1967; Cozzarelli *et al.*, 1967; Olivera & Lehman, 1967). These enzymes are similar to those from eukaryotic systems as they are all required for the basic function of resealing strand interruptions in DNA. DNA ligases act at sites of single strand breaks forming a phosphodiester bond. The assays developed for DNA ligases often directly measure phosphodiester bond formation, and the sealed DNA becomes resistant to phosphatase. An assay system of this type was utilized by Weiss *et al.* (1968)(a), (Fig. 1.1). DNA ligase activity is measured in units where one Weiss unit is defined as the amount of DNA ligase required to convert 1nmol of 5' ^{32}P-labelled phosphomonoesters to alkaline phosphatase resistant diesters in twenty minutes at 20°C.

An intermediate in the DNA ligase reaction is an enzyme-adenylate complex, in which DNA ligase is covalently bound to AMP. It is possible to isolate such

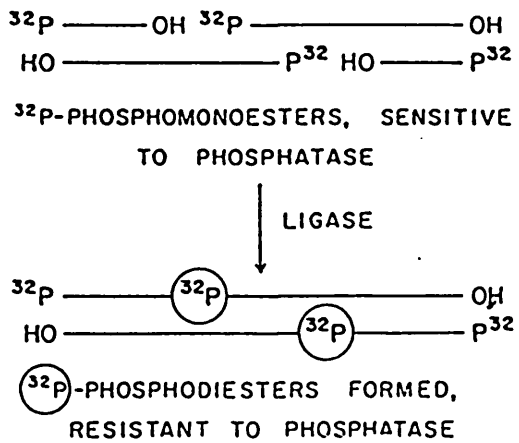


Fig. 1.1 Schematic representation of the standard assay for DNA ligase (From Weiss et al., 1968).

complexes from reactions containing DNA ligases isolated from bacteriophage T4, mammalian cells, S. cerevisiae and S. pombe, indicating the similarity between their mechanisms of action (Weiss et al., 1968(b); Soderhall & Lindahl, 1973(b); Banks & Barker, 1986). The DNA ligase activity from E. coli differs since it requires NAD rather than ATP. However, the general mechanism of action atributed to this enzyme can be applied to all DNA ligases. Hence the kinetics of this reaction in E. coli has been described by Modrich & Lehmann (1973), who found that this DNA rejoining reaction obeys ping-pong kinetics. The rate of the DNA ligase catalysed diphosphopyridine nucleotide-nicotinamide exchange reaction is unaffected by NH_4^+, indicating that the activation occurs at steps subsequent to the formation of the ligase-adenylate complex. This exchange reaction is faster than the rate of DNA joining, thus demonstrating that ligase-adenylate can be formed at a rate sufficient to be an intermediate in the overall reaction. The proposed model of the mechanism of action in E. coli and T4 DNA ligase can be seen in Fig. 1.2.

Teraoka and Tsukada (1982) have purified a large active fragment of mammalian DNA ligase I and performed kinetic analysis on this polypeptide, using 'nicked' DNA as a substrate. The results of the initial velocity and product inhibition studies indicated that the ligase reaction proceded through a uni-uni- uni-bi, ping pong

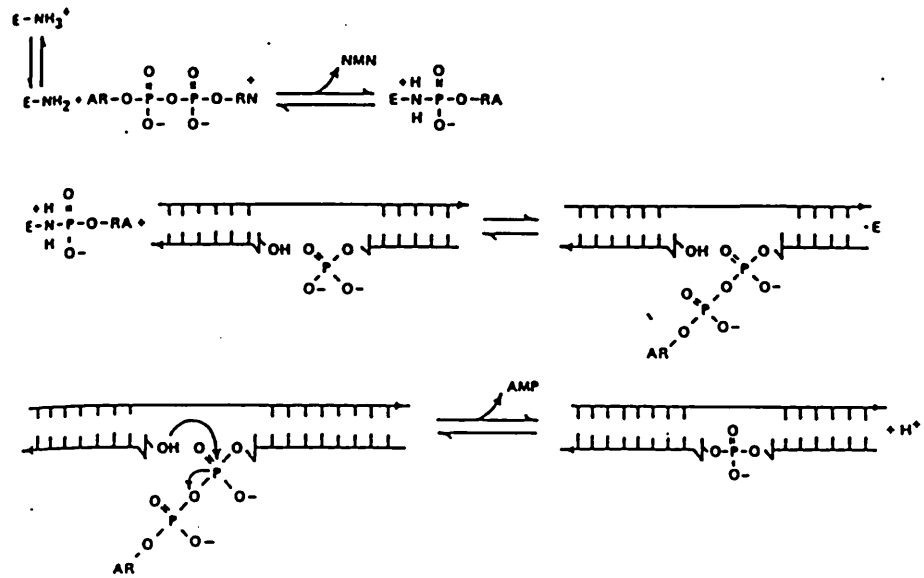


Fig. 1.2 Mechanisms of action of E.coli DNA ligase

The first step in this reaction is the formation of an enzyme intermediate by the transfer of an adenylate group of the co-enzyme to the εNH_2 of a lysine residue of the enzyme. This is followed by the transfer of the adenyl group from the enzyme to the 5′ phosphoryl terminus thus activating the adenyl group. Finally, the phosphodiester bond is formed by attack of the 3′ hydroxyl terminus of the DNA on the activated 5′ phosphoryl group with the release of AMP (from Modrich & Lehman, 1973).

mechanism in a similar fashion to the enzymes purified from E.coli and bacteriophage T4. The order of the substrate addition and release was, ATP, PPi, nicked DNA, sealed DNA and 5'-AMP (Fig. 1.3). A DNA ligase-adenylate complex in which AMP was bound in a pyrophosphate linkage to the 5' phosphorylated end of the DNA was also found. However, it was not demonstrated whether this complex was a significant intermediate in the main reaction pathway. Although T4 and E.coli DNA ligase have the same mechanism of action, the T4 enzyme was shown to exhibit an additional reaction by Fareed & Richardson (1970). This enzyme is capable of utilizing hybrids of ribose and deoxyribose homopolymers as a substrate, however, the rate of reaction with this substrate was much slower than the reaction with the homopolymer substrate. The authors suggested that this type of reaction may occur in vivo where such an activity could be utilized in regions of the T4 genome in which mRNA is transcribed, and single strand breaks could appear either in the mRNA or in the DNA strand being transcribed.

1.3 Functions of DNA ligases In Vivo

An insight into the physiological role of DNA ligases has been obtained from the study of conditional lethal DNA ligase mutants of E. coli, S. cerevisiae and S. pombe. These mutants have demonstrated that DNA ligase is the only enzyme responsible for sealing DNA chains in

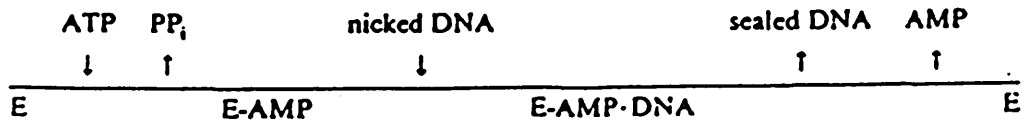


Fig. 1.3. Mechanism of action of mammalian DNA ligases The reaction proceeds through the uni-uni uni-bi ping-pong mechanism (from Teraoka et al., 1983).

replication, repair and recombination.

1.31 Functions of DNA Ligases in Replication

The requirement for a DNA ligase activity in replication processes was first demonstrated in a report by Okazaki *et al*. (1968). These authors found that DNA may be replicated discontinuously in short segments which are subsequently joined into the continuous strands that make up the chromosome. In such a discontinuous replication system, a DNA ligase activity would be required for the joining of nascent pieces and this enzyme must form an integral part of the cellular replication machinery.

Three DNA ligase mutants of *E. coli*, *lig*4, *lig*ts7 (Gottesman *et al*., 1973) *lig*321 (Horiuchi *et al*., 1974) and mutations in the DNA ligase genes of either *Schizosaccharomyces pombe* or *Saccharomyces cerevisiae* (*cdc*17 and *cdc*9 respectively, Nasmyth, 1977; Johnson & Nasmyth, 1978) all accumulate Okazaki fragments at the restrictive temperature, thereby demonstrating that DNA ligases are required to join nascent pieces of DNA. These mutants all have heat sensitive DNA ligase activities, although the severity of the defect is variable. Thus, the *E. coli* mutant *lig*321 is most affected and cell death occurs rapidly at 42°C due to degradation of cellular DNA. The mutants *lig*ts7 and *lig*4 have 1% DNA ligase activity at 42°C. The effect of this reduction is not immediately

lethal, indicating that all the remaining cellular DNA ligase is probably being utilized for replication and consequently the repair and recombination functions along the chromosome must be neglected.

In rapidly proliferating mammalian cells, DNA ligase I is the major species, comprising up to 90% of the total activity. Furthermore, in regenerating rat liver there is a fifteen fold increase in DNA ligase I activity, but no parallel increase in DNA ligase II (Soderhall, 1976). These two lines of evidence suggest that only one of the two enzymes (DNA ligase I) may be required for essential DNA replication events.

1.32 Function of DNA Ligases in DNA Repair

The role that DNA ligase carries out in the repair of damaged DNA is demonstrable by the effects that DNA damaging agents have upon DNA ligase mutants of *E. coli*, *S. cerevisiae* and *S. pombe*.

The *E. coli* DNA ligase mutants *lig*ts7 and *lig*4 show increased cellular sensitivity to ultraviolet (uv) (Pauling & Hamm, 1968; Gottesman *et al.*, 1973; Konrad *et al.*, 1973), ionizing radiation (Dean & Pauling, 1970), and alkylating agents (Gottesman *et al.*, 1973; Konrad *et al.*, 1973). Incubation of *lig*ts7 cells, after UV irradiation, at the restrictive temperature, markedly decreases the extent of repair of single strand breaks, hence indicating that DNA ligase is required for the completion of the

excision repair process (Young & Smith, 1977).

S. cerevisiae cdc9 and S. pombe cdc17 mutants are more sensitive to killing at the restrictive temperature after exposure to UV light (Nasmyth, 1977; Johnston & Nasmyth, 1978) and methylmethane sulphonate (MMS) (Johnston, 1979) than wild type cells. Sensitivity to killing by DNA damaging agents depends upon the severity of the ligase defect. The DNA ligase mutant cdc9-9 is deficient in joining single strand breaks produced after gamma (γ) irradiation (Moore, 1982), whereas the survival of cdc9-1 cells is not significantly different from wild type (Johnston, 1979). It has been suggested that DNA ligase II may function in maintaining the integrity of DNA, such that this enzyme would be responsible for sealing strand breaks caused by DNA damaging agents (Soderhall, 1976). A reaction sequence where DNA ligase II induction occurs after damage to DNA has been proposed (Creissen & Shall, 1982), but there has been little further evidence to support this theory.

1.33 Functions of DNA Ligases in Recombination

1.33 i) Hyper-rec Phenotype

Konrad (1977) isolated E.coli mutants that exhibited enhanced recombination between a pair of chromosomal duplications. Four of these mutants were found to have a defect in a late step in Okazaki fragment joining and contained a defective DNA ligase activity. In addition, when S. cerevisiae cdc9 DNA ligase mutants are held at the restrictive temperature prior to plating at the permissive temperature, there is an increase in both intragenic and intergenic recombination. It was proposed that single-strand breaks left open in DNA synthesized at the restrictive temperature may lead to increased recombination (Game et al., 1979). To investigate how the DNA ligase deficiency in S. cerevisiae cdc9 mutants might effect recombination, Fabre & Roman (1978) utilized a system where a conversion event in a heteroallelic diploid would lead to the formation of a wild type gene. For this process to occur, two reaction are necessary; a break in the gene which is followed by a ligation step. A shift in growth temperature of the cdc9 mutants from 22°C to 35°C prevented gene conversion and the authors therefore concluded that a functional DNA ligase must be a requirement for recombination. The finding that there is an increase in recombination in E. coli and S. cerevisiae DNA ligase mutants can be easily reconciled, as most

models of this process exhibit DNA strand breaks at an early stage (Radding, 1978), therefore, subsequent ligation is probably required in order to complete recombination. From the behaviour of these hyper-rec mutants it may be inferred that the concentration of nicked, gapped, or single stranded DNA is rate limiting in recombination. The mechanism of recombination in bacteria and yeast may be analogous to sister chromatid exchange (SCE) in mammalian cells since both processes involve homologous recombination. Therefore, these events may utilise similar reaction pathways.

1.33 ii) Sister chromatid exchanges

Sister chromatid exchanges (SCE) occur as a consequence of switching and exchange of DNA during replication processes. A replication defect may alter the spontaneous incidence of SCE in the cell following the introduction of DNA lesions by external agents. There are three factors which influence the frequency of SCE; the number and type of DNA lesions induced in a cell; the ability of the cell to remove and correctly repair lesions prior to DNA replication and the efficiency of the normal DNA replication machinery (Evans, 1982). In healthy individuals, although the normal processes of DNA replication and repair involve the concerted actions of a wide range of enzymes, including topoisomerases, gyrases, nucleases, polymerases and ligases, there is a very small

difference in the frequency of spontaneous SCE, which ranges from 3-8 per cell (Crossen et al., 1977). Orgel (1963) has proposed that the SCE frequency should increase with increasing age, but neither Galloway & Evans (1972) nor Crossen (1977) have found such a correlation.

There are several inheritable cancer prone syndromes which, at the cellular level, exhibit elevated amounts of chromosomal aberrations and an increased frequency of SCE. Since SCE occurs during DNA replication (Ishii & Bender, 1980) it is possible that cell lines derived from these syndromes are deficient in a replicative enzyme, such as a DNA ligase.

1.4 Bloom's syndrome

A number of diseases such as ataxia telangiectasia, Bloom's syndrome, Cockayne's syndrome, Fanconi's anaemia, Freidreich's ataxia, Werner's syndrome and xeroderma pigmentosum all show chromosomal instability and are often referred to as the chromosomal breakage syndromes (Ray & German, 1983). Table 1.1. shows that cell lines derived from certain diseases such as ataxia telangiectasia, Fanconi's anaemia and xeroderma pigmentosum exhibit extreme sensitivities to specific DNA damaging agents namely, γ-irradiation, DNA crosslinking agents and UV-irradiation respectively. This indicates that the biochemical defects associated with these syndromes may be

Table 1.1

Disease	Cellular Sensitivities	Cellular Characteristics
Ataxia telangiectasia	Extreme sensitivity to γ irradiation (Taylor, 1978, Lewis et al., 1979) bleomycin and neocarzinostatin (Shiloh & Becker, 1982).	Exposure of cells to γ irradiation and γ-mimetic agents causes gross chromosomal abberations (Taylor, 1982; Painter, 1985; Ejima & Sasaki, 1986).
Bloom's syndrome	Slight sensitivity to UV-irradiation of 215 and 254nm (Giannelli et al., 1977; Ishizaki et al., 1980; Zbinder & Cerutti, 1980; Hirshi et al., 1981; Hansen et al., 1980), mitomycin C (Ishizaki et al., 1980), bromodeoxyuridine (Yamamoto & Fujiwara, 1986) and ethylmethansulphonate (EMS) (Krepinsky et al., 1979).	Cells show an elevated frequency of sister chromatid exchanges (SCE), 10-15 times that found in normal cells (Chaganti et al., 1974; German et al., 1974; German et al., 1977).
46BR	Extreme senstivity to 3-amino benzamide (Teo et al., 1983b) slight sensitivity to γ and 254nm UV-irradiation, mitomycin C, MMS, EMS, MNNG, MNU (Teo et al., 1983a).	Normal frequency of sister chromatid exchanges (SCE), (Teo et al., 1983b).
Cockayne's syndrome	Sensitivity to UV (Marshall et al., 1980)	Sister chromatid exchanges induced by exposure of cells to UV (Marshall et al., 1980).
Fanconi's anaemia	Sensitive to killing by the DNA crosslinking agents, mitomycin C, nitrogen mustard longwave UV + 8 methoxypsoralen (Sasaki & Tonomura, 1973).	Increased number of spontaneous chromosomal abberations (Friedberg et al., 1979; Kato & Stich, 1976; Auerbach & Wolman, 1976) Also an increased SCE frequency after treatment with mutagenic and carcinogenic agents (Sasaki & Tonomura, 1973).
Friederich's ataxia	Slightly sensitive to ionizing radiation (Bridges & Harden, 1982), sensitive to mitomycin C and nitrogen mustard (Evans et al., 1982).	
Werner's syndrome	Normal sensitivity x-ray and UV killing (Fujiwara et al., 1977).	Normal SCE frequency, but some clones of cells have rearranged chromosomes (Hoehn et al., 1975).
Xeroderma pigmentosum	Sensitive to killing by UV, UV-mimetic chemicals, (Cleaver & Bootsma, 1975)	There is an increased SCE frequency following exposure of these cells to UV.

related to their inability to repair lesions caused by such compounds. However, cell lines derived from Bloom's syndrome patients are slightly sensitive to killing by a variety of DNA damaging agents, suggesting that the enzyme or enzymes which may be deficient in this disease occur in a common step, which is required for the repair of damage caused by each of these compounds, for example DNA polymerase or DNA ligase.

1.41 SCE in Bloom's syndrome cell lines

Cell lines derived from Bloom's syndrome patients have a spontaneous frequency of SCE greater than 12-fold that found in cell lines from normal individuals (Chaganti et al., 1974), with the frequency of SCE ranging from 40-160. This high SCE frequency in Bloom's syndrome cell lines is widely regarded to be a hallmark of the disease, however, a number of reports demonstrate that this is not always the case. For example, in Bloom's syndrome lymphoblastoid cell lines established by transformation with Epstein-Barr Virus (EBV), Hashimoto et al.,(1983) and Hashimoto & Sukenaza (1984) have observed two cell populations, one with a high frequency of SCE (~100-140 per cell), whilst the other had nearly normal levels of SCE. In cells with a near normal number of SCE, the frequency was reduced 10 fold from the high levels observed before transformation to 10 SCE per cell.

In addition, Shiraishi et al. (1983) have found three types of B-lymphoid cells from Bloom's syndrome patients, increased SCE and increased chromosomal aberration, increased SCE without chromosomal aberrations and normal levels of SCE with chromosome aberrations. There are two possible explanations for these results; either that EBV immortalization normalized both the level of Bloom's syndrome SCE and chromosome aberrations or that there are two cell populations in vivo.

In the Bloom's syndrome cell lines used in the experiments described in this thesis, two cell types were studied; those with a high SCE of 60-100 per cell and those with lower SCE of 8-15 per cell, which still represens an increased number of SCE compared to controls, which exhibit SCE of 3-6 per cell.

1.42 Biochemical characteristics of Bloom's syndrome

Several enzymes involved in both DNA repair and replication have been studied in Bloom's syndrome. Abnormal regulation of uracil-DNA glycosylase has been reported in some Bloom's syndrome fibroblasts (Gupta & Sirover, 1984; Yamamoto & Fujiwara, 1986). In synchronized cell cultures, levels of this enzyme reach a peak a few hours before DNA synthesis. In a study of two Bloom's syndrome cell lines, Yamamoto and Fujiwara (1986) found a delay in the induction of this enzyme. The authors claim that the the high SCE frequency in Bloom's

syndrome fibroblasts is due to sensitivity of these cells to bromodeoxyuridine caused by the abnormal regulation of uracil-DNA glycosylase. This is unlikely for two reasons, firstly bromodeoxyuridine degrades to ring-fragmented derivatives but not uracil, thus uracil-DNA glycosylase is not required for repair and secondly, in one Bloom's syndrome line the regulation of this enzyme was normal. Cells from this syndrome are not deficient in O^6-methylguanine DNA methyltransferase or hypoxanthine DNA glycosylase, but in these cases it was also claimed that the regulation of the two enzymes is abnormal (Leim et al., 1986; Dehaza & Sirover, 1986).

Bloom's syndrome cells have a slower rate of DNA synthesis, and this has indicated that these cells may be deficient in a replication intermediate. They exhibit a 20% reduced rate of DNA chain growth (Hand & German, 1977) which was confirmed by Kapp (1982) who reported a 35% reduction in rate. Furthermore, Ockey and Saffhilll (1986) have shown that Bloom's syndrome fibroblasts show a slower rate of DNA synthesis and DNA maturation than control cells. Giannelli et al. (1977) reported that Bloom's syndrome fibroblasts are abnormally sensitive to UV in vitro owing to a deficiency in an enzyme involved in a post incision event. Coupled with the delayed maturation of DNA chains, this indicated that the metabolic defect in Bloom's syndrome includes a step important for both DNA synthesis and repair. However,

this defect is not caused by a deficiency in DNA polymerase α or β as these activities when assayed in Bloom's syndrome fibroblasts were found to be normal (Parker and Lieberman, 1977; Spanos et al., 1986).

1.43 Clinical Characteristics

This syndrome was first described by Bloom (1954) as "Congenital telangiectatic erythemia resembling lupus erythomatosus in Dwarfs. Probably a syndrome entity". He subsequently identified three clinical "cardinal features" that are associated with this disease. The most apparent of these features is stunted growth, but this is not a pituitary dwarfism and no evidence of thyrotrophic, or adenocorticotropic deficiency has been found in any of the cases. In this syndrome, the children are small at birth and their growth rate is relatively slow. The average heights of affected individual males and females are 130cm and 120cm respectively (Bloom, 1966). Patients with this disease also have congenital telangiectasia erythema which is seen as butterfly regions on the cheeks, and of the nose, margins of the eyelids lips, forehead and ears. Sun sensitivity is the third clinical symptom of this disease. This manifests itself as a erythema of the face following exposure to sunlight (Bloom, 1966). It has been observed that there is a greater ratio of males to female with this disease (Bloom, 1966; German et al., 1983) of 1.4. This is possibly either due to a higher death rate of females

with this syndrome during foetal and post natal life. Alternatively, the skin lesions in females tend to be considerably milder and under diagnosis could be the reason for the apparently greater number of males with this disease. A possible example of under diagnosis of a female patient with Bloom's syndrome is provided by the cell strain 46BR derived from a patient with hypogammaglobulinaemia. This individual exhibited the three cardinal features that are associated with Bloom's syndrome (Bloom, 1966). Thus, the patient has a dwarfed appearance, venous dilation of the skin which could be due to congenital telangiectatic erythema, and although sun sensitivity was not reported, cells from this patient were hypersensitive to both γ and 254 UV irradiation (Teo et al., 1983a). This patient, as has also been found with some Bloom's syndrome patients, had a selective IgA deficiency. Cells from this patient, like those derived from Bloom's syndrome patients, were hypersensitive to a wide range of DNA damaging agents (Table 1.1). Thus, this cell line was sensitive to mitomycin C and to the alkylating agents dimethylsulphate, ethylmethanesulphonate, methylmethane sulphonate, N-methyl-N'-nitro-N-nitroso-guanidine and N-methyl N-nitrosourea (Teo et al., 1983a) and 3 amino-benzamide (Teo et al., 1983b). The main difference observed between Bloom's syndrome cells and 46BR can be found in their SCE frequencies. In Bloom's syndrome the high SCE frequency

is a hallmark of this disease, whereas in 46BR the SCE frequency was found to be close to normal (Table 1.2). However, as has been discussed previously, a number of reports show that not all Bloom's syndrome cell lines exhibit markedly elevated SCE frequencies and in some patients, a near normal level has been found.

Immunological examinations of patients with Bloom's syndrome has also revealed decreased serum levels of at least one class of immunoglobulins IgG, IgM or IgA (Landau et al., 1966; Schoer et al., 1967; Huttervoth et al., 1975). Tahiguchi et al. (1982) reported that the in vitro generation of immunoglobulin producing cells was impaired. Further experiments suggested that the B-cell differentiation ability and T cell regulatory function for the generation of immunoglobulin producing cells was incompletely developed in these patients.

Bloom's syndrome patients also have a predisposition to malignant neoplasms. In a survey by German et al. (1983), out of the 103 Bloom's syndrome patients originally studied, only 80 remained with an average age of 18.2 years. There were 28 malignant neoplasms detected at a mean age of 20.7 yrs. A variety of cancers were found including leukemia, Wilm's tumour, colon cancer and adeno-gastro-oesophaegal cancer.

1.44 Ethnic origins of Bloom's syndrome patients

The Bloom's syndrome gene is most common in the

Table 1.2

Cell line	Cell type	Ethnic origin of patient comments	SCE frequency (per cell)	Source
GM3403C	lymphoblast	Ashkenazi Jew	50-60	Human Genetic Mutant Cell Repository
GM3402C	fibroblast	Ashkenazi Jew same patient as GM3403C	50-60	Human Genetic Mutant Cell Repository
GM5289A	fibroblast	Japanese	N/A	Human Genetic Mutant Cell Repository
GM8505	fibroblast	Ashkenazi Jew	N/A	Human Genetic Mutant Cell Repository
1032	lymphoblast	French Canadian	60	R. Weksberg
1004	lymphoblast	Mennonite	80	R. Weksberg
W674	lymphoblast	Ashkenazi Jew	60-80	E. Henderson
AA8751	lymphoblast	Ashkenazi Jew	10-15	E. Henderson
AA874	lymphoblast	Ashkenzai Jew parent of AA8751	3-8	E. Henderson
D8612	lymphoblast	Ashkenazi Jew	10-15	E. Henderson
46BR	fibroblast	Anglo-Saxon	3-8	A.R. Lehmann

Jewish population and has a particularly high frequency amongst the Ashkenazim of 0.0042, thereby implying a heterozygote frequency greater than 1 in 120. Analysis of the ancestral origin of the patients with Bloom's syndrome has indicated that the Ashkenazi gene originated from an individual who lived in Eastern Europe centuries ago (German, 1969)(Fig. 1.4). In over half of the patients of non-Jewish ethnic origin, with an affected child, there was consanguinity of parents, indicating that in a Western population a recessive autosomal gene is probably responsible for this syndrome. In this study several cell lines derived from non-Jewish Bloom's syndrome patients were used (Table 1.2). The Mennonite patient from which the cell line W1004 was derived comes from a small isolated community and it is possible that this mutant arose independently in a similar fashion to the Ashkenazi Bloom's syndrome mutation.

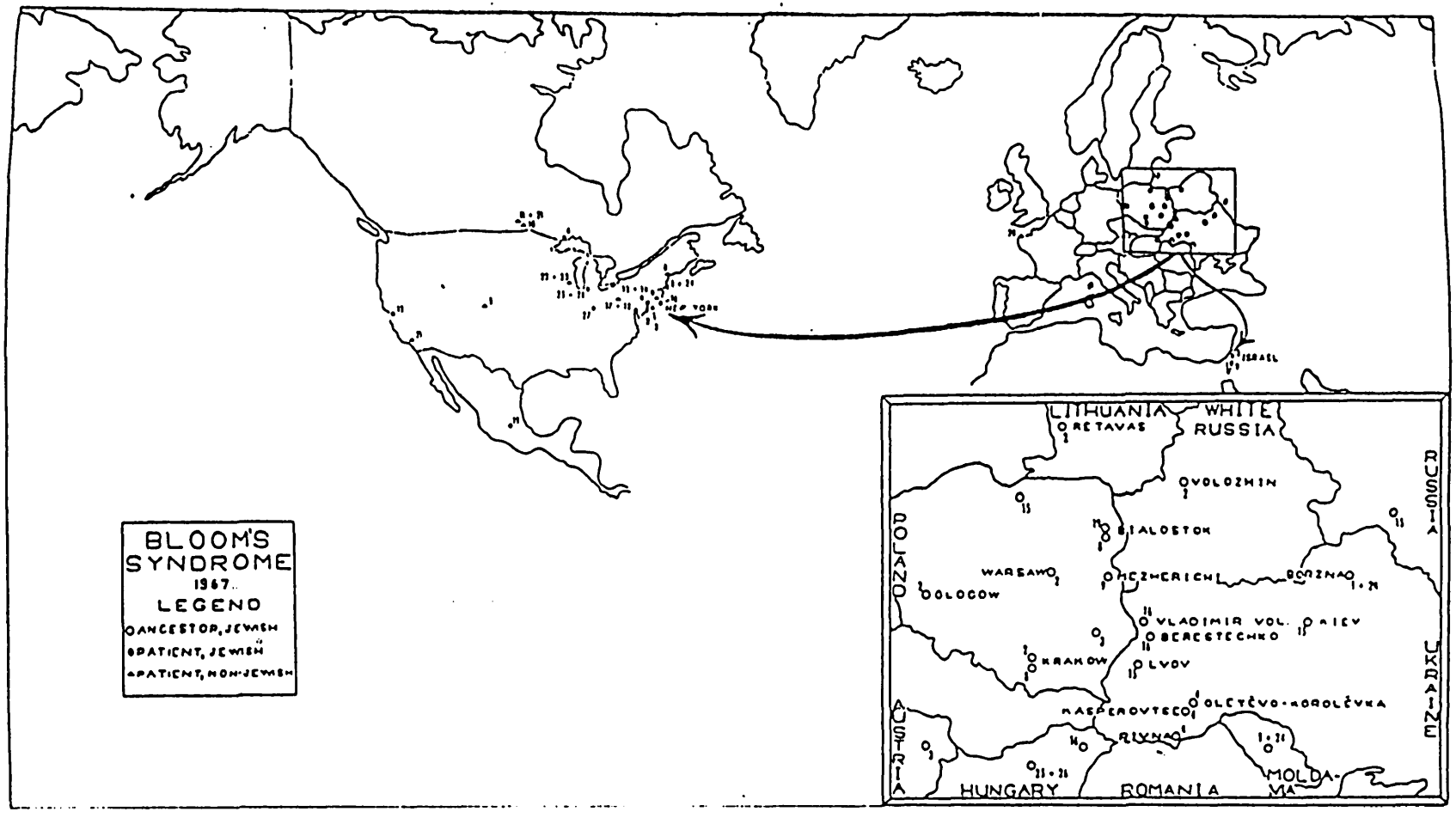


Fig. 1.4 Map showing places of residence of recognized affected individuals
In addition geographical origins of the Jewish are represented. The insert, enlarging Eastern Europe, shows ancestral towns (from German, 1969)

Chapter 2

Materials and Methods

Materials and Methods

2.1 Chemicals

2.11 Sources

The chemicals, co-enzymes and commercial preparations of enzymes and DNAs used in this study were purchased from Sigma (London) Ltd., Boehringer Manneheim Corporation (London), Pharmacia (Uppsala, Sweden) and New England Biolabs. All other laboratory reagents and solvents of analytical grade were purchased from BDH Chemicals (Poole, Dorset) and Fisons Ltd. (Leicester). All radiochemicals were purchased from Amersham (Buckinghamshire).

2.2 Substrates

2.21 Homopolymer Substrate

Oligo dT_{25-30} (Pharmacia) was 5'-end labelled by incubating with T4 polynucleotide kinase in the presence of γ^{32}PATP, in a reaction mixture containing 2μm cold ATP, 50mM Tris.HCl pH7.6, 10mM $MgCl_2$, 5mMDTT, 100μCi ^{32}P ATP(3000 Ci/mmol). The labelled ^{32}P oligo dT_{25-30} was separated from the unincorporated triphosphates by spinning the reaction mixture through a Sephadex G50 column (Maniatis et al., 1982). The ^{32}P oligo dT was anealed to either poly(dA) or

poly(rA) by heating a 1:1 mixture of polymer:oligo(dT) to 90°C for 10min followed by cooling to room temperature.

2.22 Nicked Circular DNA

Plasmid DNA pAT153 (Twigg & Sherrat, 1980) was isolated from Escherichia coli K12 by standard techniques described by Maniatis et al. (1982). Nicked circular DNA was prepared by EcoRI cleavage of the plasmid in the presence of ethidium bromide (Parker et al., 1977), in a reaction mixture containing 30μg DNA, 30 units of EcoRI, 0.35mg ml^{-1} ethidium bromide, 10mM Tris-HCl pH7.5, 5mM $MgCl_2$, 100mM NaCl, 5mM DTT and 0.02% Triton X-100 at 37°C for 1h.

2.23 Blunt-end DNA

A blunt-ended DNA substrate was prepared by the cleavage of ϕX174 replicative form DNA (obtained from BRL) with HpaI in a reaction mixture containing 40μg DNA, 400 units of HPAI, 20mM Tris.HCl buffer pH7.5; 50mM NaCl, 5mm DTT, 5mm $MgCl_2$, 0.02% triton X-100. The reaction was incubated at 37°C for 1h and three fragments of 3.7kb, 1.2kb and 0.4kb were obtained.

2.24 Antibody directed against DNA ligase 1

The rabbit antibody directed against 1000-fold purified DNA ligase I used in the work was obtained as described by Soderhall & Lindahl (1975).

2.3 Enzyme Assays

2.31 Alcohol dehydrogenase (EC.1.1.1.1.) was assayed by following the reduction of NAD at 360nm. The reaction mixture contained: 3M aqueous ethanol, 0.2ml; 5mM NAD, 0.1ml and enzyme preparation made up to final volume of 3ml with phosphate buffer (pH8.5).

2.32 Alkaline phosphatase (E.C.3.1.3.1.) was assayed by determining the increase in absorbance at 410nm from the hydrolysis of p-nitrophenylphosphate to p-nitrophenol at 25°C. Reaction volume of 3ml contained, 0.5M Tris.HCl pH8.0 and 0.003M p-nitrophenylphosphate.

2.33 Catalase (E.C.1.11.1.6) activity was assayed by following the enzyme-catalyzed decomposition of H_2O_2 at 240nm. An H_2O_2 solution was freshly prepared [0.16μl of 30% (v/v) H_2O_2 in 100μl of 0.1M potassium phosphate buffer, pH7.0] to give an initial A_{240} of 0.5. To 3ml of this solution enzyme was added.

2.34 0^6-Methylguanine DNA methyltransferase (E.C.2.1.1.63)

These assays measure the repair of 0^6-methylguanine (Demple et al., 1982). The substrate heat depurinated alkylated DNA (2000 cpm) containing 0^6-MeGua residues was incubated with crude cell extracts in 100ml of standard reaction mixture (70mM Hepes.KOH buffer; pH7.6, 1mM EDTA; 10mM DTT) for 15 min at 37°C. The reaction was stopped by chilling the assay mixture on ice and the DNA and protein precipitated by the addition of an equal volume 0.8M TCA. After 5min at 0°C, the samples were microfuged and the supernatants discarded. To each pellet 100mM HCl (150μl) was added and the mixture was heated to 70°C for 30 mins. This treatment liberates unrepaired 0^6-MeGua from DNA in the free form whilst DNA and proteins (including the methylated form of the transferase) remain precipitated (Karran et al., 1978). Following centrifugation the supernatants were recovered and their radioactivity determined.

2.35 DNA ligase (E.C.6.5.1.1.).

All DNA ligase assays were carried out in a reaction mixture of 50μl containing 50mM Tris.HCl pH7.8; 10mM $MgCl_2$; 5mM DTT; 1mM ATP and 50μg ml^{-1} nuclease-free bovine serum albumin.

2.351 Polydeoxynucleotide Substrate

To determine DNa ligase activity, samples were

incubated with the substrate for 1h at 16°C in a reaction mixture containing 50μl of standard ligase buffer and 3-5ng (~5000cpm) of either [^{32}P] oligo(dT)·Poly(dA) or [^{32}P] oligo (dT).poly(rA). The reaction products were then heated to 90°C for 10min, cooled on ice and to each reaction mixture 1 unit of calf intestinal phosphatase added. This enzyme removes 5'-^{32}P labelled phosphomonoesters from unligated oligonucleotide substrate. The reaction products were precipitated by the addition of 10μg of herring sperm DNA and 500μl of ice cold 5% (w/v) TCA. The reaction tubes were left on ice for 10min and the resulting precipitates collected on nitrocellulose filters (45μm, Schleicher & Schuell, BA 85) and the radioactivity of the precipitates measured in a liquid scintillation counter (Packard) using toluene and 0.5% Permablend III as a scintillant. This assay measures ligase activity in terms of phosphatase-resistant radioactive material. One ligase unit is the amount of enzyme required to form 1nmol of phosphatase resistant phosphate in twenty minutes at 20°C (Weiss et al., 1968).

2.352 Blunt-ended DNA

DNA ligase assays with blunt-ended DNA substrate were carried out in reaction mixture of 40μl standard ligase buffer containing 0.5μg DNA, 17.5% w/v polyethylene glycol 6000 and 1.5×10^{-6} units of DNA ligase. The incubation was for 1h at 37°C. The reaction products were separated by gel

electrophoresis on a 0.8% (w/v) agarose gel containing 5μg ml^{-1} ethidium bromide and visualized under ultraviolet light transillumination.

2.353 Nicked Substrates

Reaction mixtures (20μl) contained 0.2μg of nicked DNA, standard ligase buffer and $1.5x10^{-6}$ units of DNA ligase. Reactions were allowed to proceed for 1h at 16°C and were terminated by extraction with buffered pH8.0 phenol:chloroform (1:1 vol/vol). The reaction products were subsequently characterized by agarose gel electrophoresis.

2.4 Phosphodiester bond formation

In order to demonstrate that the alkaline phosphatase resistance of the radioactive phosphate residues in the polymer substrate (2.21) treated with mammalian DNA ligase I or II was due to the formation of 3′-5′ phosphodiester bonds, the substrates were degraded to mononucleotides by the subsequent action of micrococcal nuclease and spleen phosphodiesterase (Weiss et al., 1968). The mononucleotides obtained were separated by high pressure liquid chromatography HPLC using a Partisal SAX column (Whatman, 4.6x25cm) and the solvent system described by Saffhill and Hall (1981).

2.5 Enzyme purification techniques

2.51 Column chromatography

Columns were obtained from Pharmacia Ltd. (Uppsala, Sweden) and Biorad (England). Column eluent was run through a Pharmacia UVI detector system linked to a Pharmacia Rec 421 recorder, and the unit set to monitor protein absorbance at 280 nm. Fractions were collected in a Redirac LKB Instruments fraction collector. Protein absorbance of major peaks were confirmed manually by measuring A_{280} using a Perkin-Elmer lambda 6 spectrophotometer. All procedures were performed at 0-4°C unless otherwise stated.

2.52 Whole Tissues.

Fresh calf thymus was obtained from Quantock Veal, Dorchester.

2.53 Purification of DNA ligase I and Ligase II.

The connective tissue and fat was removed from 600g of fresh calf thymus gland, which was subsequently washed with ice cold phosphate buffered saline. The calf thymus was then homogenized (in a Moulinex blender) in 3 litres of extraction buffer (0.1m NaCl, 50mM Tris.HCl pH7.5, 10mM 2-mercaptoethanol, 1mM EDTA, 0.5mM phenylmethylsulfonyl-fluoride and 0.5 $\mu g\ ml^{-1}$ each of the protease inhibitors pepstatin, leupeptin and chymostatin) and left with gentle

stirring for 1h. The homogenate was centrifuged at 6000 rpm for 20min and nucleic acids were removed from the supernatant by precipitation by the slow addition of neutralized polyethyleneimine ('Polymin P' supplied by BDH) to a final concentration of 0.5% (v/v) under continuous stirring. After 30min the mixture was centrifuged to pellet the precipitated DNA and the supernatant recovered. Finely ground $(NH_4)_2SO_4$ was added slowly to give 42% saturation, and the protein precipitate removed by centrifugation. To the supernatant additional $(NH_4)_2SO_4$ was added to give 67% saturation and left on ice for 30min. The mixture was centrifuged and the resulting protein pellet was redissolved in buffer A, composed of 50mM NaCl, 50mM Tris.HCl (pH7.2), 10mM 2-mercaptoethanol, 1mM K_2HPO_4, and 1mM EDTA. The solution was dialysed against this buffer for 16h. The dialysate (360ml) was applied to two parallel phosphocellulose columns (Whatman), 40x30cm previously equilibrated with buffer A. The columns were initially washed to remove unbound protein and the DNA ligase I and II eluted stepwise with buffer A containing 0.5M NaCl, but without EDTA. Fractions of 10ml were collected at a flow rate of 80ml h^{-1}. DNA ligase I and II activities which co-chromatographed in this step were then applied directly to a hydroxyapatite column (HA-Ultrogel, LKB Products) at a flow rate of 50ml h^{-1} (2.5x18cm) and eluted with a linear gradient (800ml) of 1 to 200mM K_2HPO_4 in buffer B (0.5m NaCl; 50mM Tris.HCL pH7.5 and 10mM 2-mercaptoethanol)

collecting 6ml fractions. This procedure separated the DNA ligase I and II activities. The two DNA ligase activities were then purified further by anion exchange chromatography. DNA ligase I (24.5ml) was dialysed twice for 18h against 5L of buffer C (15mM KH_2 PO_4, pH7.2, 1mM EDTA and 10mM MSH and the dialysate loaded onto a phosphocellulose column (1.8cmx16cm) previously equilibrated with the same buffer mixture. The column was washed at a flow rate of 40ml h^- and the DNA ligase I activity eluted with a buffer containing a linear gradient (800ml) of 0-400mM NaCl collecting 5ml fractions. The peak of DNA ligase I activity (20ml) was brought to 72% saturation by the addition of solid $(NH_4)_2SO_4$. The precipitate was pelleted by centrifugation and the protein redissolved in 1ml of buffer B and loaded onto a Sephadex G-150 column (100x1cm). The protein was eluted at a flow rate of $1ml h^{-1}$, collecting 0.5ml fractions.

The DNA ligase II fraction from the hydroxyapatite step (57ml) was concentrated by the addition of $(NH_4)_2SO_4$ to 72% saturation. The precipitate was pelleted by centrifugation and the protein pellet redissolved in buffer B loaded onto a Sephadex G-150 column (100x1cm), and eluted under the same conditions as described for DNA ligase I. Molecular weights of DNA ligase I and II were estimated by the method of Andrews (1964). Active fractions of each enzyme were pooled, concentrated 3-fold by dialysis against buffer (50mM Tris·HCl pH7.5, 10mM 2-mercaptoethanol, 1mM EDTA) containing 50%

glycerol, and stored at -20°C. In agreement with previous results (Soderhall & Lindahl, 1975) DNA ligase I was purified by approximately 1000-fold, while DNA ligase II was purified 200-fold. A summary of the purification procedure is given in Table 2.1.

2.54 Sucrose Gradient Density Centrifugation

Linear sucrose gradients containing 5-20% sucrose, 200mM NaCl, 50mM Tris-HCl buffer (pH7.5), 10mM 2-mercaptoethanol, 1mM EDTA were prepared and left to equilibrate overnight at 4°C. Cell extracts (as described in section 2.61) volume 150μl containing ~3mg protein were layered carefully onto the gradients, which were then placed in a SW50.1 rotor and centrifuged for 10h at 40,000 rpm at 4°C in a Beckman 8 ultracentrifuge. The molecular weight markers used were apoferritin (440,000), alcohol dehydrogenase (150,000), catalase (232,000), alkaline phosphatase (80,000) and carbonic anhydrase (29,000). Catalase was also used as an internal reference in all gradients. Fractions (200μl) were collected and assayed for DNA ligase activity. The molecular weight of the activity peaks were calculated according to the method of Monty & Siegel (1966).

2.55 Fast Pressure Liquid Chromatography

The FPLC system was obtained from Pharmacia Ltd. (Uppsala, Sweden). The human DNA ligases I and II were

Table 2.1

Fraction	Protein (mg/ml)	Specific Activity (units/mg)	Total Activity Units	Purification	Yield (%)
Crude cell extract	16	$2x10^{-5}$	0.96	0	100%
$(NH_4)_2\ SO_4$	7	$2x10^{-4}$	0.62	11	65%
Phosphocellulose	1	$1.3x10^{-3}$	0.43	65	42%
Hydroxyapatite I	2.8	$5.9x10^{-3}$	0.31	250	30%
II	0.2	$2.2x10^{-3}$	0.022	115	23%
Phosphocellulose DNA ligase I	1.4	$1.4x10^{-2}$	0.25	600	19%
Sephadex G150 DNA Ligase I	1	$2x10^{-2}$	0.12	1000	12%
DNA ligase II	1.2	$5x10^{-3}$	0.015	200	15%

separated according to their size (Soderhall & Lindahl, 1973) on a prepacked superose 12 column (30cm x 2cm). Cell free extracts were prepared as described in section 2.61 and 2-4mg of samples in a volume of 100μl were loaded on to the column. The proteins were eluted at a flow rate of 0.4ml min^{-1} collecting 200μl fractions into test tubes (precooled on ice). The fractions were assayed for ligase activity as described in section 2.351.

2.56 Thermal Inactivation of Enzymes

Pooled, size fractionated peaks of DNA ligase I or II (0.1-0.2mg protein per ml) were incubated at 50°C ($\pm$0.1°C) in a Braun thermomix 1420 waterbath (Braun Ltd., Mebunzen, West Germany). Aliquots were removed at predetermined time intervals, cooled immediately and stored on ice for 30min. The samples were then microfuged to remove any precipitated proteins, and assayed for DNA ligase activity.

2.57 Protein Determination

Protein concentration was measured by the method of Bradford (1976) using bovine serum albumin as the standard.

2.58 Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis was performed by the method of Laemmli (1970) on 1mm thick vertical slab gels (20.5cmx16cm), containing 3-10% (w/v) acrylamide in the resolving gels

prepared from the following stock.

(i) Resolving gel 10% acrylamide

Acrylamide/bisacrylamide 30:0.8(w/v)	10ml
1m Tris-HCl (pH8.8)	11.2ml
20% (w.v) sodium dodecylsulphate	0.15ml
Distilled H_2O	8.7ml
Total volume	**30ml**

The resolving gels were chemically polymerized by the addition of 200μl of ammonium persulphate (10%w/v) and 20μl of TEMED, to 30ml of the acrylamide mixture under a water-saturated butan-1-ol overlay. After polymerisation, the butan-1-ol was removed and the gel surface washed twice with distilled water. A 5% (w/v) acrylamide stacking gel was then layered on top of the resolving gel. This gel was prepared from the following stock solution.

(ii) Stacking gel (5% acrylamide)

Acrylamide/bisacrylamide 30:0.8(w/v)	1.67ml
1M Tris.HCl buffer (pH6.8)	1.25ml
20% (w/v) sodium dodecylsulphate	0.05ml
Distilled H_2O	7.03ml

The stacking gel was chemically polymerised by the addition of 50ml of $(NH_4)_2SO_4$ (10%w/v) and 10μl TEMED. The reservoir buffer contained 0.025M Tris base, 0.192M glycine (pH8.3) and 0.1% (w/v) SDS. The sample buffer contained 0.25M Tris.HCl buffer (pH6.8), 40% (v/v) glycerol, 5% (w/v) 2-mercaptoethanol, 2% (w/v) SDS and 0.005% (w/v) bromophenol blue and was added in the ratio of 1 part sample buffer to 3 parts of sample, prior to loading the protein samples on the gel. Samples were prepared by heating for 3min in a boiling water bath. Electrophoresis was performed at a current of 30mA for 5-6h or overnight at 8mA until the bromophenol blue tracking dye migrated to the bottom of the gel.

Molecular weight determinations of proteins were determined by SDS-PAGE using a Biorad protein molecular weight calibration kit containing myosin (200kDa), β-galactosidase (116kDa), phosphorylase B(92kDa), bovine serum albumin (66.2kDa) and ovalbumin (45,kDa).

Protein was detected by staining the gels for 6h with 0.2% (w/v) Comassie Brillant Blue R250 dissolved in a solvent system consisting of methanol-water-acetic acid (5:5:1 by vol.). Gels were diffusion destained by repeated washing in the above solvent mixture, lacking the dye.

2.59 Western Transfer

Gels were placed on nitrocellulose filters and the protein transfered using a Biorad apparatus at 30V for 10h

(Burnett, 1981). The transfer buffer contained methanol 20% (v/v), 0.025M Tris·HCl and 0.192M glycine (pH8.8). The nitrocellulose was then incubated for 10min at 25°C with phosphate buffered saline containing 0.05% Nonidet P40 and 10% horse serum (v/v). Rabbit antiserum directed against DNA ligase I (15μl) (Soderhall & Lindahl 1975) was added and the incubation maintained for 10h at 10°C. The serum was removed from the filter and the filter washed 3 times with phosphate buffered saline containing 0.05% Nonidet P_{40}, and 10% (v/v) horse serum was added to the nitrocellulose, to block the protein sites that had not reacted with the antibody. Secondary antibody 20μl (goat anti rabbit IgG peroxidase conjugated, purchased from Nordia Immunologicals) was added to the blocking buffer and incubated for 2h at 4°C. The filter was washed with phosphate buffered saline to remove any unbound or secondary antibody and the Western blot was developed by the addition of a solution containing 1mg/ml β chloronaphthol, and 8μl H_2O_2 in 15ml of phosphate buffered saline.

2.510 Identification of Enzyme-adenosine monophosphate complexes

The procedure followed was that of Banks & Barker (1985). Cell extracts from human tissue culture cells were made as described in (2.61). These extracts were incubated with 50μl reaction mixture containing 50mM Tris-HCl (pH7.8),

10mM $MgCl_2$, 5mM DTT, 40μCi α ^{32}P ATP (3000 Ci mmol^{-1}, Amersham) and 2μm ATP. The reaction was incubated for 20min on ice and stopped by the addition of 30μl of 50% (w/v) TCA and 3.75% sodium pyrophosphate (w/v). The precipitate formed after 30min was microfuged for five minutes and the resulting pellet washed with 7% (w/v) TCA and 1% sodium pyrophosphate. The pellet was redissolved in 25μl of lysis sample buffer (2.58) and boiled for 2min before loading the samples onto a 8% polyacrylamide gel. Following electrophoresis, the gels were either stained with Comassie blue R250 and autoradiographed using Kodak (XAR5) film, or transferred to nitrocellulose and probed with antibody directed against DNA ligase I as described in 2.59.

2.6 Purification of DNA ligase I and II from cultured human cells

2.61 Preparation of cell free extracts

The cells ($5x10^7$ - $1x10^8$) were collected by centrifugation and washed twice in phosphate buffered saline. The pellet was resuspended in 300μl of ice cold extraction buffer (100mM NaCl, 50mM Tris.HCl, pH7.5, 10mM β-mercaptoethanol; 1mM EDTA; 23μg/ml^{-1} aprotinin and 0.5μg/ml^{-1} each of pepstatin, leupeptin and chymostatin) and disrupted in a hand held homogenizer. The crude cell extract

was left on ice for 1h and then centrifuged in a Microfuge for 10min to remove the unbroken cells and cellular debris. Nucleic acids were precipitated by adjusting the NaCl concentration to 0.2M and the addition of 'Polymin P' to 0.5% (v/v). This mixture was microfuged after 30min and the resulting precipitate discarded.

2.62 Partial purification of DNA ligase I and II from Raji cells

Cells from the cell line Raji were harvested at two stages of the cell cycle, at the stationary phase with $1x10^6$ cells ml^{-1}, and when the cells were growing exponentially with $7x10^5$ cells ml^{-1}. Cell pellets of approximately 8g (wet weight) were obtained from 12 litres of cell culture from cells in the exponential phase of growth and 8 litres of culture from cells in the stationary phase of growth. Cell free extracts were made as described in section 2.61 and all other purification procedures were followed as in section 2.52, except that column sizes were scaled down, such that the phosphocellulose column size was 1.5x4cm and the hydroxyapatite column size was 1x3cm.

2.63 Partial purification of DNA ligases I and II from xeroderma Pigmentosum cells group A (line GM2250) and Bloom's sydrome cells (line GM3403C)

Cell pellets, (~2g), were obtained from 2 litres of

culture from each of two cell lines. Cell extracts were made and nucleic acids removed as described in section (2.61). To the crude cell extracts solid $(NH_4)_2SO_4$ was added to 70% saturation and the resulting pellet redissolved in 0.5M NaCl, 50mM Tris HCl (pH7.5), 10mM 2-mercaptoethanol and 1mM EDTA (buffer A). The extracts were then loaded onto two AcA-34 columns, 80x1cm, equilibrated with buffer A and 0.5ml fractions were collected. From each cell line, the fractions containing DNA ligase I and II activity were pooled separately and dialysed against buffer containing 5mM KCl, 50mM Tris·HCl (pH7.5), 10mM 2-mercaptoethanol and 1mM EDTA (buffer B). The dialysates were loaded onto DNA cellulose columns, 4cm x 1cm, previously equlibrated with buffer B and protein eluted stepwise with buffer B containing 50mM KCl, 100mM KCl and 200mM KCl respectively. Fractions were assayed for DNA ligase activity, the active fractions were pooled and dialysed against 50mM Tris·HCl (pH7.8), 10mM 2-mercaptoethanol, 1mM EDTA and 50% (v/v) glycerol and then stored frozen at -20°C.

2.7 Cell Culture

2.71 Cell lines

The cell lines derived from patients with Bloom's syndrome, GM3403C, GM3402C, GM8505 and GM5289 were obtained from the Human Genetic Mutant Cell Repository, Camden, New

Jersey. The Bloom's syndrome cell lines, W674, D8612, AA874 and AA875-1 were kindly provided by Dr. E. Henderson (Henderson & Ribecky, 1980). The cell lines 1032 and 1004 derived from patients clinically diagnosed as Bloom's syndrome, were made available by Dr. R. Weksberg, Hospital for Sick Children, Toronto. The cell line 46BR was obtained from Dr. A. Lehmann, Cell Mutation Unit, University of Sussex (Webster et al., 1982). All other cell lines derived from patients with various inheritable syndromes, that is, ataxia telangiectasia GM1526, Cockaynes's syndrome GM1712, Fanconis anaemia GM4510, xeroderma pigmentosum group A, GM2250, group D GM2253, variant GM2449, Werners syndrome AG3829 and the control cell lines GM730, GM1953, GM0612 were purchased from the Human Genetic Mutant Cell Repository, Camden, New Jersey.

2.72 Growth media

Lymphoblastoid cells were grown at 37°C in Roswell Park Memorial Institute media (RPMI) supplemented with 15% foetal calf serum (FCS) in 1 litre spinner flasks. Fibroblast cell lines were grown in Dulbecco's modified Eagle's medium (E4) also supplemented with 15% (v/v) FCS, at 37°C in a humidified atmosphere containing 10% CO_2.

2.73 Sister chromatid exchange

The number of SCEs were determined by established methods (Perry & Wolf, 1974). These experiments were carried out by

Dr. D. Sheer, ICRF, Lincoln's Inn Fields, London.

2.74 Cell Survival

The sensitivity of the Bloom's syndrome fibroblast cell line GM3402C to various damaging agents was tested by two different methods: i) Exponentially growing cells were removed from plates by trypsinization and re-seeded in 90mm plastic dishes (Sterilin) at appropriate low densities. Cells were allowed to attach for 9h and were incubated with varying concentrations of the drug under test. The plates were left for three weeks and the number of colonies formed counted (Arlett, 1981). ii) The method of Goss & Parsons (1977) was also used. In this procedure the fibroblast cell lines either GM730 or GM3402C were plated at 10^4 cells per well in a 6 well dish (Sterilin). Varying concentrations of the drug under test were added to the medium and the plates were incubated for 5-8 days. After this period the cells were washed with phosphate buffered saline and replenished with fresh medium containing [methyl ^{3}H] thymidine (Amersham PLC), 43 Ci/μmole at a concentration of 5μ Ci/ml. Following a 4h incubation period, the medium was removed from the plates and the wells washed three times with phosphate buffered saline to eliminate any unincorporated ^{3}H-thymidine. The cells were removed from the plates by trypsinization and filtered onto glass fibre filters (Whatman) with extensive washing. The radioactivity of the cells collected on the filters was determined in a

liquid scintillation counter; the rate of uptake of thymidine correlates directly with the number of cells surviving the treatment with the agent.

2.75 Transfer of plasmid DNA into human fibroblast cell lines

Three methods of transfecting the cells were used: i) calcium phosphate technique of Wigler et al. (1979). Cells of line GM3402C were seeded at $2x10^5$ and $1x10^6$ cells per 90mm plate. Calcium phosphate precipitates were formed by the addition of 140mM NaCl, 20mM Hepes-KOH (pH7.2), and 1mM K_2HPO_4, 240μl, to a mixture of an equal volume of 20μg plasmid DNA (PAW1 20μg or PSV2NEO 20μg) in a buffer containing 10mM Tris·HCl (pH7.5), 1mM EDTA, 150mM NaCl and 250mM $CaCl_2$. The precipitate formed by the addition of these two mixtures was added to the cells in 1ml of fresh medium and the plates incubated for 5-16h. After this period, the medium was removed and the plates washed twice with buffer containing NaCl, KCl, Na_2HPO_4 and 1% glucose. To each plate 2ml of 20% (v/v) dimethylsulphoxide (DMSO) was added for 2min. The plates were rewashed and left for 48h in non-selective medium, Dulbecco's modified Eagles medium with 15% FCS (v/v), after which 400μg/ml geneticin (G418) (Gibco) plate was added to select for the gene coding for neomycin resistance.

ii) Kawai & Nishizawa (1984) method using a polycation. The polycation used was polybrene (Aldrich chemical Co., Milwakee, Wis., U.S.A.). Cells $1x10^6$ per 90mm dish were

incubated with 3ml of complete medium, 5μg/ml of polybrene and 20μg of DNA (PAW1 or PSV2 NEO) for 6h at 37°C. The medium was then removed and 5ml of 20% (v/v) DMSO added in serum free medium for 2min. The plates were washed twice with serum free medium and incubated with complete medium for 48h before the addition of 400μl of geneticin (G418)(Gibco).

iii) Electroporation technique as described by Potter et al. (1984) and Chu et al. (1987). In these methods cells ($3x10^6$) were resuspended in 1ml of buffer containing 20mM Hepes (pH7.05), 137mM NaCl, 5mM KCl, 0.7mM Na_2HPO_4, 6mM dextrose and 20μg/ml of plasmid DNA (either PAW1 or PSV2NEO). The cells were then exposed to two pulses of a single voltage with 10s interval between pulses. The electric field used were 3kv/cm, 6kv/cm and 7.5kv/cm. The pulsed cells were left to stand for 15-20min on ice before replating in non-selective medium, Dulbeccos modified E4 + 15% FCS. After 96h the cells were transferred to selective medium containing 400μg/ml geneticin.

2.8 Molecular Biology Techniques

2.81 Plasmid DNA

Plasmid pR12Sclig4, which contains cdc9, the Saccharomyces cerevisiae DNA ligase gene, was obtained as a purified DNA preparation from Dr. L. Johnston, National

Institute for Medical Research (Barker & Johnston, 1983). The mammalian expression vector pSV2 Neo (Southern & Berge, 1982) was from Dr. P. Karran. This plasmid encodes ampicillin resistance and G418 resistance. Plasmid pAT153 (Twigg & Sherratt, 1980) was obtained from Dr. B. Sedgwick.

2.82 Bacterial growth conditions

Bacterial strains were grown at 37°C with aeration in L-broth (Miller, 1972) (10g Bactotryptone, 5g Bacto yeast extract, and 5g NaCl per litre) supplemented with thymidine (20μg/ml) and/or antibiotics when necessary.

2.83 Transformation Procedure

Preparation and transformation of competent E.coli cells was by the calcium chloride/rubidium chloride method as described by Maniatis et al (1982).

Bacterial cultures grown to $O.D_{600}$ of 0.3 units were harvested by centrifugation at 3000 rpm for 10min at 4°C. The pellet was resuspended in 40ml of buffer containing 30mM potassium acetate (pH5.8), 100mM $RbCl_2$, 10mM $CaCl_2$ 50mM $MnCl_2$ and 15% (v/v) glycerol and left for 1h at 0°C. The bacteria were again centrifuged and the pellet redissolved in 4ml of buffer containing 10mM Mops·KOH (pH7.0), 75mM $CaCl_2$, 10mM $RbCl_2$ and 15% (v/v) glycerol and incubated for 15 min on ice. The bacteria were then aliquoted into precooled Nunc tubes and stored as frozen stock at -80°C.

The bacteria were thawed at room temperature and placed on ice. To 100μl of cells 10ng of DNA was added and the mixture was left on ice for 30min. The suspension was heated to 42°C for 90sec and returned to 0°C for a further 2min. After the addition of four volumes of L. broth the culture was grown at 37°C for 1h and then plated onto L-agar containing 50μg/ml ampicillin. This method gave 10^6-10^7 colonies/μg of super coiled DNA.

2.84 Subcloning of cdc9 into pSV2 Neo

The pR12Sc lig4 plasmid was cleaved with the restriction enzyme HindIII and SalI using conditions recommended by the enzyme supplier. This digestion yielded three DNA fragments which were resolved by electrophoresis on an 0.8% agarose gel containing 0.5μg/ml ethidium bromide and visualized under UV light. The sizes of the fragments were ~5480, 4420 and 870bp by comparison with the marker lane (a "ladder" purchased from BRL). The 4420bp fragment which encoded the yeast ligase gene, was cut out of the gel and electroeluted into TE (10mM Tris pH8.0, 1mM EDTA) (Maniatis et al., 1982). The DNA was further purified by phenolchloroform extraction and precipitation with ethanol. This fragment was resuspended in TE and digested with the restriction enzyme NspBII. This gave three fragments of ~2730, 820 and 830bp, which were separated on a 0.6% (w/v) agarose gel. The 2730bp fragment was purified from the gel matrix as described above. HindIII linkers were

attached to the 2730bp fragment by incubating with 2μg of HindIII linkers (Pharmacia) in a reaction mix of 50μl containing 50mM Tris·HCl (pH7.6), 10mM $MgCl_2$, 5mM DTT and 1mM ATP, 10 units T4 DNA ligase. The excess HindIII linkers were removed by centrifuging the reaction products through a column containing a small plug of Sephadex G25 and Sephacryl S300. The fragments with ligated HindIII linkers were then cut with HindIII to generate a HindIII site, and the small oligonucleotides were separated out by passing the mixture through a Sephadex G25, Sephacryl S300 column.

The vector PSV2 Neo was digested to completion with HindIII and then ligated with the 2733 bp fragment at a ratio of 1:5 at 16°C for 10h using T4 DNA ligase in 50μl reaction mixture containing 10 units of T4 DNA ligase, 50mM Tris·HCl pH7.5, 10mM $MgCl_2$, 5mM DTT and 1mM ATP.

2.85 Screening for recombinants

The ligation products were used to transform the E.coli K12 strain DH5. E. coli transformed with plasmid DNA were selected by plating transformation mixes on L-agar plates supplemented with 50μg/ml ampicillin.

2.86 Analysis of recombinants

Plasmids were isolated by the 'miniprep' rapid boiling method of Holmes & Quigley (1981) and Maniatis et al. (1982). The plasmids were digested to completion with BamHI and SmaI

and separated on a 0.8% (w/v) agarose gel. Those containing the cdc9 gene in the correct orientation gave three fragments of 4.6, 2.6 and 1.1kb. The new plasmid was termed pAW1.

Chapter 3

Different substrate specificities of the two mammalian DNA ligases

Different substrate specificities of the two mammalian DNA ligases

3.1 General Properties of DNA ligase I and II

In this chapter I have investigated the substrate specificities of the two mammalian DNA ligases in order to establish a specific, distinguishing assay. The procedures developed have subsequently been used to provide biochemical insight into how DNA ligases may function in the mammalian cell.

3.2 Substrate Specificities of DNA ligase I and II

3.21 Nicked substrate

Both DNA ligase I and DNA ligase II are capable of catalyzing the formation of 3'-5' phosphodiester bonds in 'nicked' DNA. This results in the conversion of nicked circular plasmid into covalently closed molecules (Fig. 3.1, lanes 4 and 6) (Section 2.2). Preincubation of the two enzymes with a polyclonal antiserum directed against DNA ligase I (Section 2.2) inhibits the ligase I activity (lane 5), but has no effect on the ligase II activity (lane 7), indicating that the two enzymes may be serologically distinct.

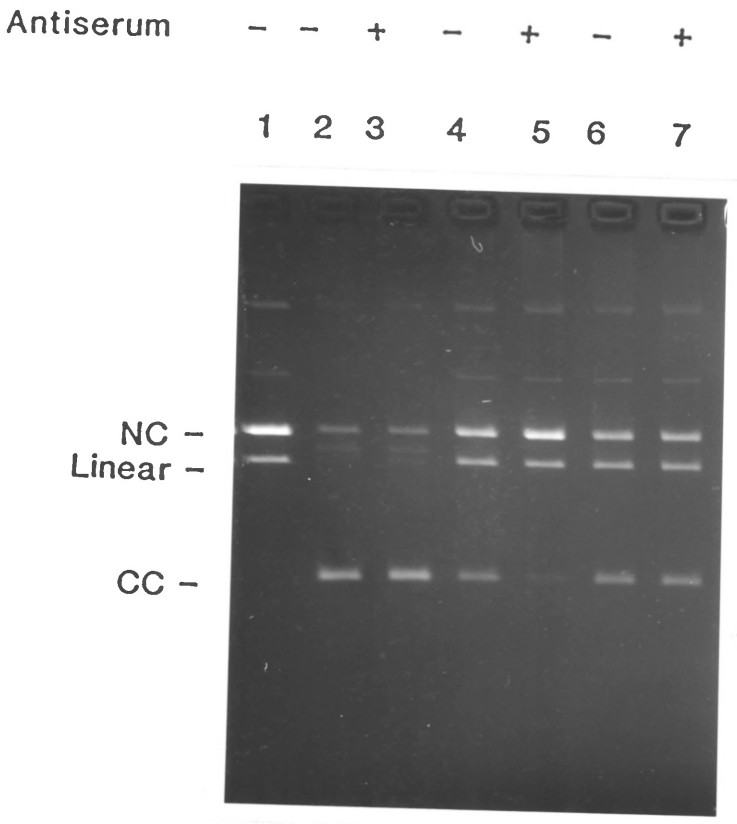


Fig. 3.1 Activity of DNA ligases under standard assay conditions in the presence of antibodies against mammalian ligase I

The conversion of nicked circular (NC) plasmid DNA to covalently closed molecules (cc) was visualized after electrophoretic separation on 1% agarose gels containing 0.5μg ml^{-1} ethidium bromide. Lane 1, no ligase added; Lanes 2 and 3, T4 DNA ligase; Lanes 4 and 5, mammalian ligase I; Lanes 6 and 7, mammalian ligase II. Ligase I antibody was included in the reactions where indicated.

3.22 Homopolymer substrate

A substrate consisting of oligomers of deoxythymidylate hydrogen bonded to polydeoxyadenylate (poly(dA)) was first used by Olivera & Lehman (1968). The rate and extent of joining of oligodeoxythymidate oligomers is dependent on two factors: 1) the length of the complementary chain and the relative molar concentrations of deoxyadenylate and deoxythymidylate residues; 2) the transitional movement of the dT oligomers relative to the poly(dA) chain during the reaction. Therefore, to obtain maximum joining of the substrate, the ^{32}[P] oligo(dT) $_{(25-30)}$ and the poly(dA) were combined in equal concentrations. At A/T ratios of 0.4 the extent of joining detected by the authors was never more than 40%, whilst at A/T ratios of >1 an imperfectly formed helix occurs also giving a low extent of joining (Fig. 3.2).

When a homopolymer substrate is used in a ligase reaction, the rate of formation of 3′ - 5′ phosphodiester bonds is also highly sensitive to temperature, perhaps because the oligo(dT) may move along the poly(dA) chains, with a faster rate of movement at higher temperatures. Although this movement improves ligation, DNA ligase II is somewhat unstable at 37°C and gives better results at lower temperatures, therefore, a temperature of 16°C was deployed for these reactions.

DNA ligase I and DNA ligase II join oligomers of deoxythymidylate$_{(25-30)}$ hydrogen bonded to polydeoxyadenylate. In these assays a limiting amount of enzyme was used and the assay was linearly dependent upon

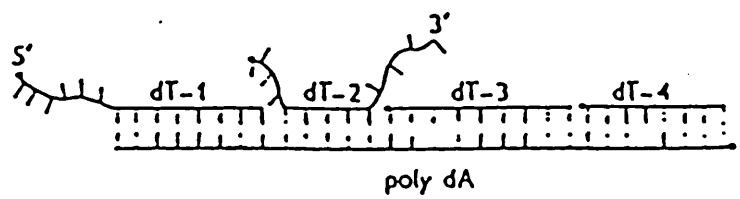


Fig. 3.2 A diagrammatic representation of the proposed structure of poly(dA)·dT formed when A/T<1
The dots represent 5′ phosphoryl termini. In the stretch of double helix shown, only the 5′-terminus of (dT-4) can react. (dT-1) cannot be (dT-2) and dT-3) cannot be joined because either the 3′- or 5′- terminus at these positions is improperly aligned.

enzyme concentration within the range 0-1.5 x 10^{-7} enzyme units for either mammalian DNA ligase I or ligase II (Fig. 3.3). Generally 3-5ng (10,000 cpm) of substrate was used per reaction.

3.23 Hybrid substrate

This substrate was made employing the same method as the homopolymer substrate (section 2.22), except that poly(rA) was substituted for poly(dA). Soderhall and Lindahl (1976) first discovered that DNA ligase II was able to act on this type of substrate, with further reports of this type of activity in mammalian cells by Bedows (1977). These initial observations have been used to develop a specific assay for DNA ligase II. The reaction of ligase II with this substrate is linear with enzyme concentration between 0-3 x 10^{-7} units and time (Fig. 3.4), up to two hours. Joining of the hybrid substrate occurs at about 50% of the rate observed with a nicked double-stranded polydeoxyribonucleotide and is comparable in this respect with the reaction that occurs with T_4 DNA ligase (Engler and Richardson, 1982). The joining activity of ligase II with oligo(dT) bound to either poly (dA) or a poly(rA) complementary chain, coeluted during both hydroxyapatite chromatography (Fig. 3.5) and gel filtration (Fig. 3.6). Furthermore, the two activities exhibited the same heat lability, 50% inactivation in 5min at 42°C and may be ascribed to the same enzyme (Fig. 3.7).

DNA ligase I does not show detectable activity with this substrate (Fig. 3.4) since reaction mixtures containing an

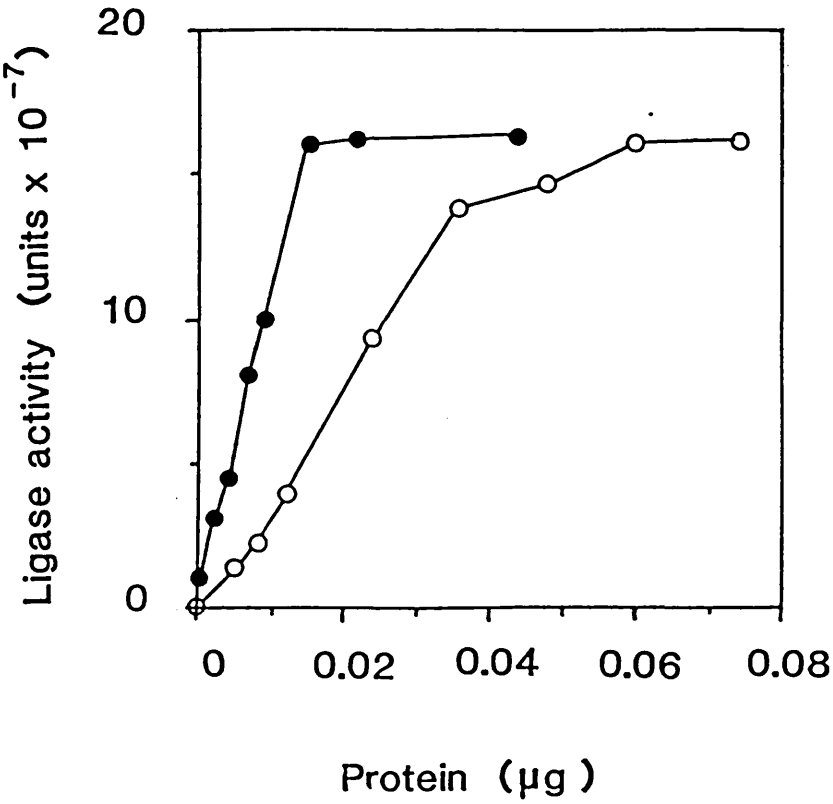


Fig. 3.3 The effect of increasing concentrations of mammalian DNA ligases on poly(dA)·oligo(dT) substrate

DNA ligase I (●) and DNA ligase II (o) were incubated with poly(dA)·oligo(dT) substrate under standard reaction conditions for 1h at 16°C.

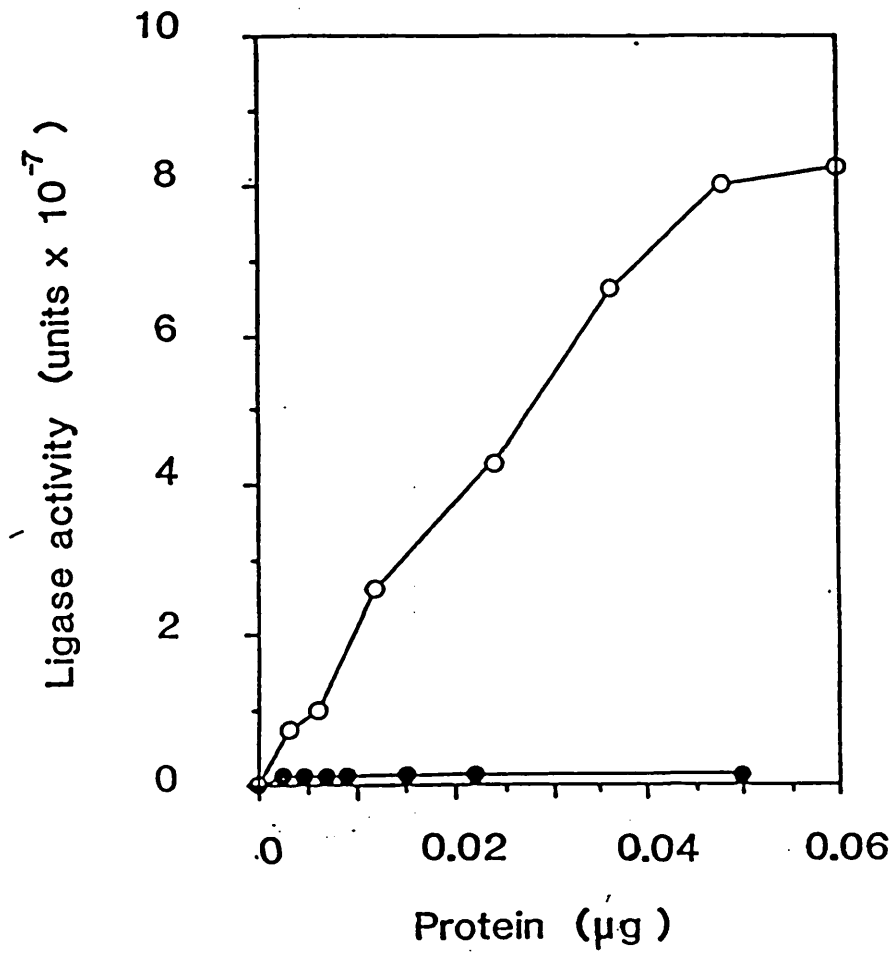


Fig. 3.4 The effect of increasing concentrations of mammalian DNA ligases on poly(rA)·oligo(dT) substrates

DNA ligase I (●) and DNA ligase II (o) were incubated with poly(rA)·oligo(dT) substrate understandard reaction conditions for 1h at 16°C.

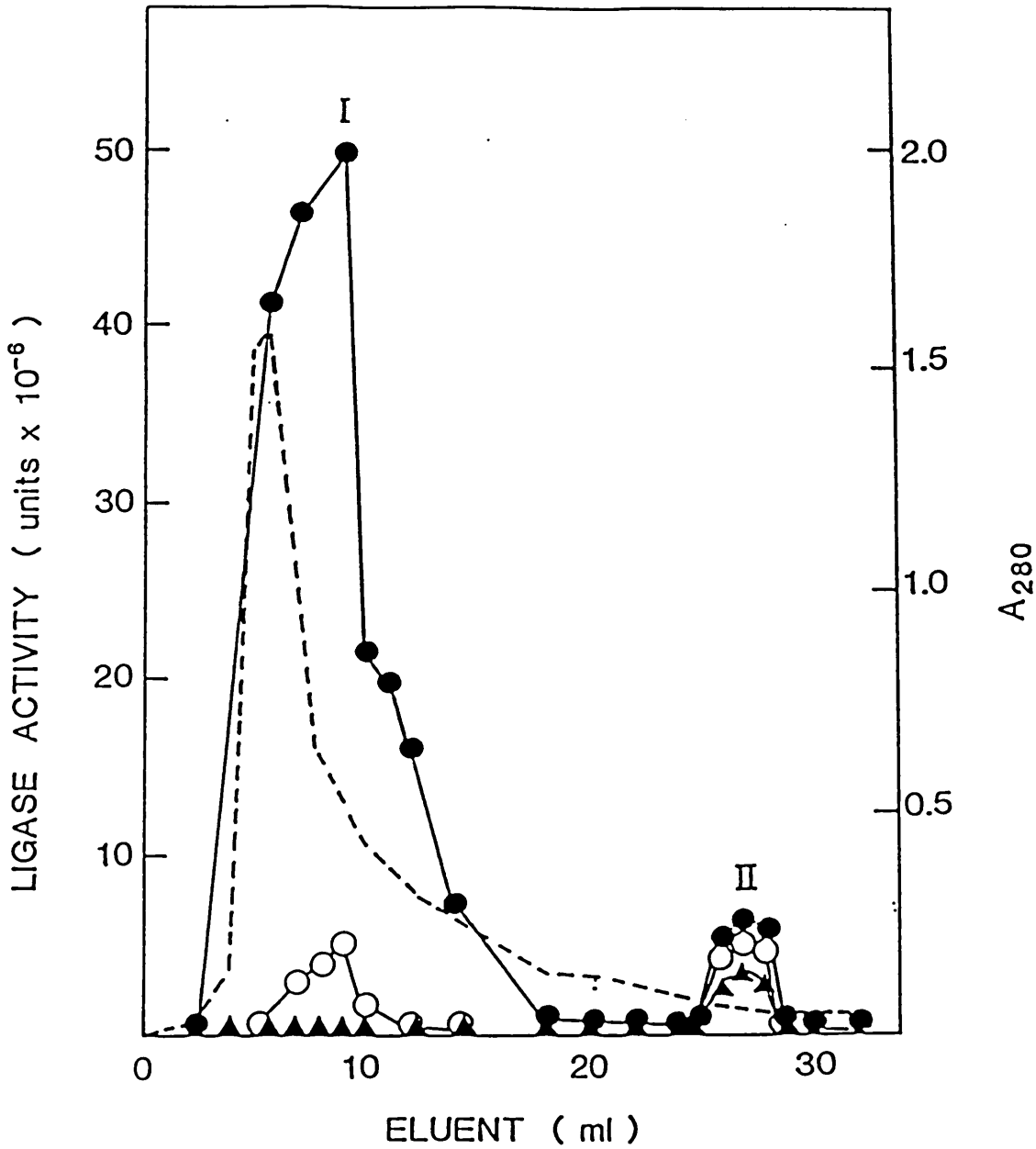


Fig. 3.5 **Assays of mammalian DNA ligases I and II with a hybrid substrate**

The two calf thymus DNA ligases (from 50g of tissue, material partly purified by phosphocellulose chromatography) were separated by hydroxapatite gradient chromatography and column fractions (5-μl aliquots) were assayed directly with poly(dA)·oligo ([5'-^{32}P]dT) (♦) or poly(rA)·oligo dT (▲) substrates. Reactions with the poly(dA)·oligo(dT) substrate were also performed in the presence of antibodies to ligase I (o). Dashed line represents A_{280}.

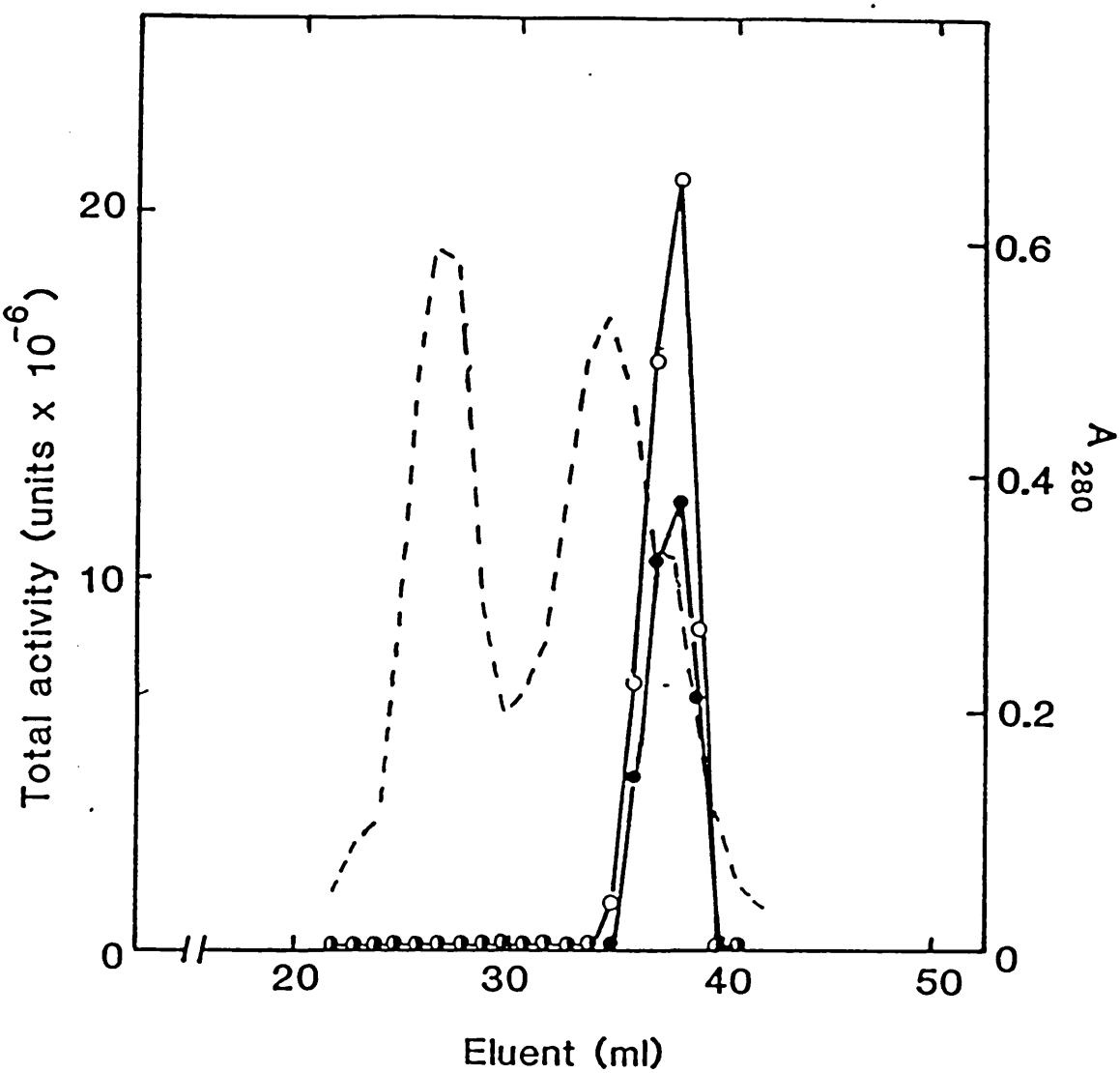


Fig. 3.6 Gel filtration chromatography of DNA ligase II

DNA ligase II (separated from ligase I by hydroxyapatite gradient chromatography) was eluted from a Sephadex G150 column and assayed with poly(dA)·oligo ([5′-^{32}P]dT) (○) and poly(rA)·oligo(dT) (●) substrates. Dashed line represents A_{280} (see section 2.53).

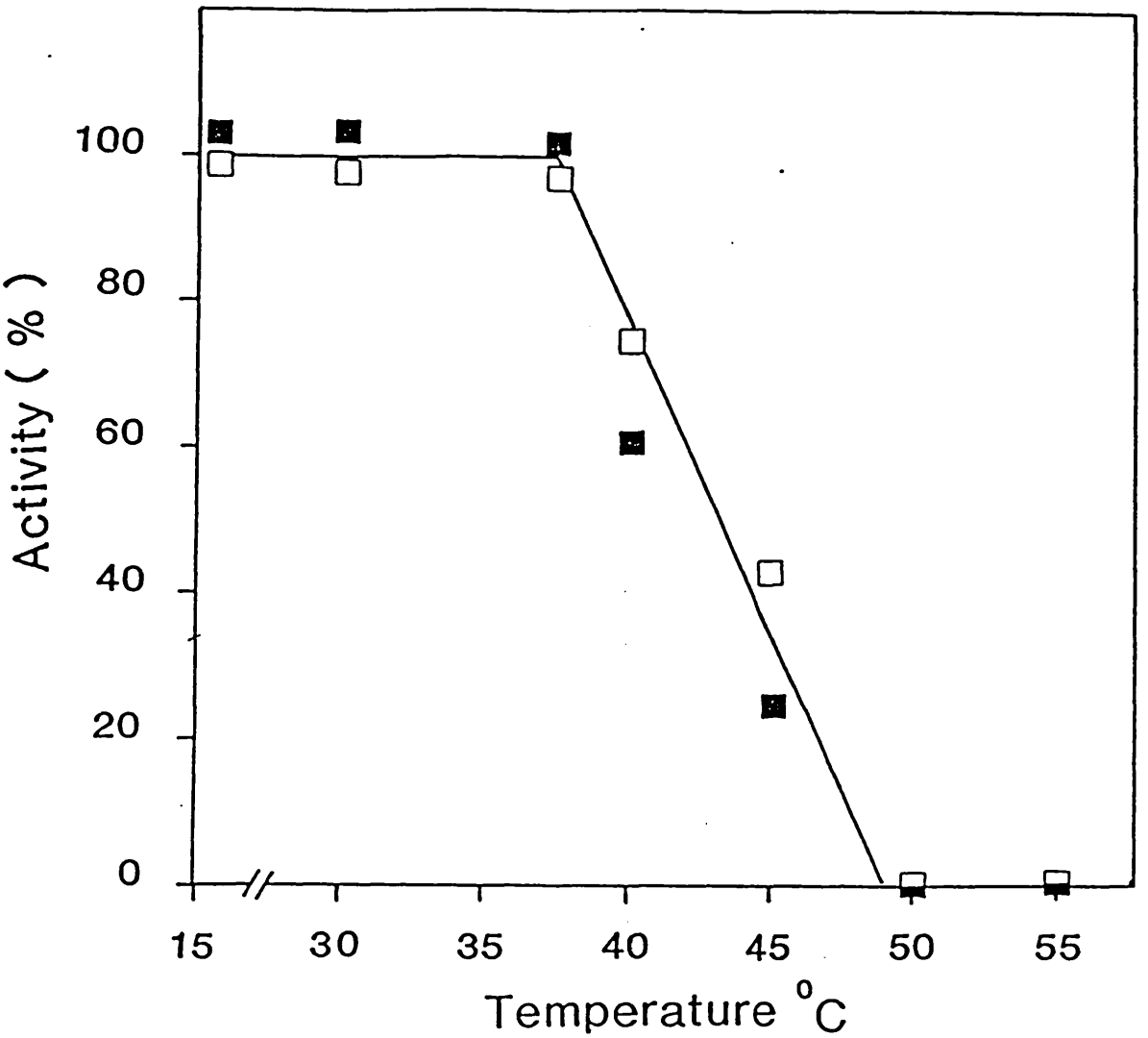


Fig. 3.7 Heat inactivation of DNA ligase II
Mammalian DNA ligase II was heated at predetermined temperatures for 5min. Aliquots (5μl) were subsequently removed and assayed for ligase activity using either poly(dA)·oligo(dT) substrate (□) or poly rA·oligo(dT) substrate (■).

excess of DNA ligase I ($1.5x10^{-6}$ units), 10 fold over that required for maximal joining of poly(dA).oligo(dT) substrate yielded no detectable joining (<5%) of an equivalent amount of poly(rA).oligo(dT) substrate. This lack of DNA ligase I activity was not due to the degradation of this substrate by contaminating RNAse H (Hausen, 1970), since mixtures of DNA ligase I preparation and the T4 DNA ligase were as active as the T4 enzyme alone.

3.24 Formation of phosphodiester bonds

The formation of phosphodiester bonds was investigated by a ^{32}P transfer reaction. Ligation reactions were carried out by incubating poly(dA)·oligo [5′-^{32} P]dT and poly(rA)·oligo [5′-^{32}P]dT with DNA ligase I and ligase II respectively. After treatment of the substrates with phosphatase, they were degraded to mononucleotides by the subsequent action of micrococcal nuclease and spleen phosphodiesterase (see section 2.4). Generation of 3′ - 5′ phosphodiester bonds between ([5-^{32}P]dT) moieties hydrogen bonded to poly(dA) or poly(rA), by the enzymes, would allow the recovery of [3′-^{32}P]dTMP. The reaction between DNA ligase I and the poly(dA)·oligo ([5′-^{32}P]dT) substrate and DNA ligase II and the poly·rA oligo ([5′ ^{32}P]dT) yielded more than 90% of the phosphatase resistant radioactive material as 3′-dTMP (Fig. 3.8). These data show that both DNA ligase I and DNA ligase II act as DNA ligases and that DNA ligase II generated phosphodiester bonds with the poly(rA)·oligo(dT) substrate.

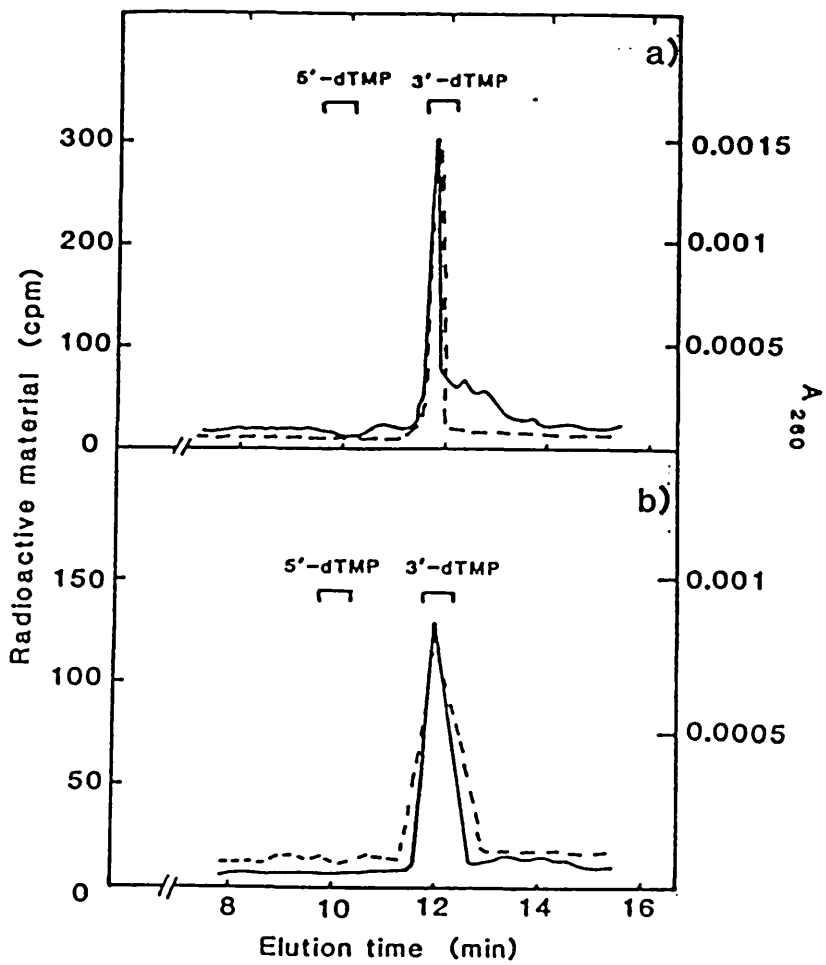


Fig. 3.8 **HPLC analysis of ligation reaction procedures** Poly(dA)·oligo(dT) and poly(rA)·oligo(dT) substrate were incubated with DNA ligase I (a) or DNA ligase II (b) respectively. The reaction products were digested by the subsequent action of micrococal nuclease and spleen phosphodiesterase and separated by reverse phase HPLC. Bold line represents A_{260} and dashed line radioactive material.

3.25 Km of DNA ligases for ATP

It has been shown previously that DNA ligase I and II exhibit different Km for ATP (Soderhall & Lindahl, 1976). In agreement with previously published results, the Km's for ATP of DNA ligase I and DNA ligase II were found to be ~$5x10^{-7}$ and $1x10^{-4}$ respectively (fig. 3.9). Therefore, in the standard DNA ligase reaction mixtures used in experiments presented here, there was an excess of ATP ($1x10^{-3}$M).

3.26 Blunt-end joining of DNA

In addition to joining single strand breaks in duplex DNA, several DNA ligases, from different sources, are capable of sealing strand breaks between two fully base-paired termini of DNA duplexes, i.e., blunt ended DNA. This function is less efficient than the sealing of single-strand interuptions, but can be increased by at least two orders of magnitude by utilizing conditions which employ high concentrations of a variety of macromolecules such as polyethylene glycol (PEG) or Ficoll. These compounds appear to act by increasing the apparent overall concentration of reactants and are often referred to as volume excluders. In the presence of such "volume excluders" DNA ligase from rat liver nuclei, E. coli and Thermus thermophilus HB8 have been found to catalyse the blunt-ended ligation of DNA very actively, whereas no detectable blunt-end ligation was found in the absence of the volume excluders (Zimmerman & Pheiffer, 1983; Takahashi & Uchida, 1986).

All reactions with blunt-ended DNA (section 2.35.2) were

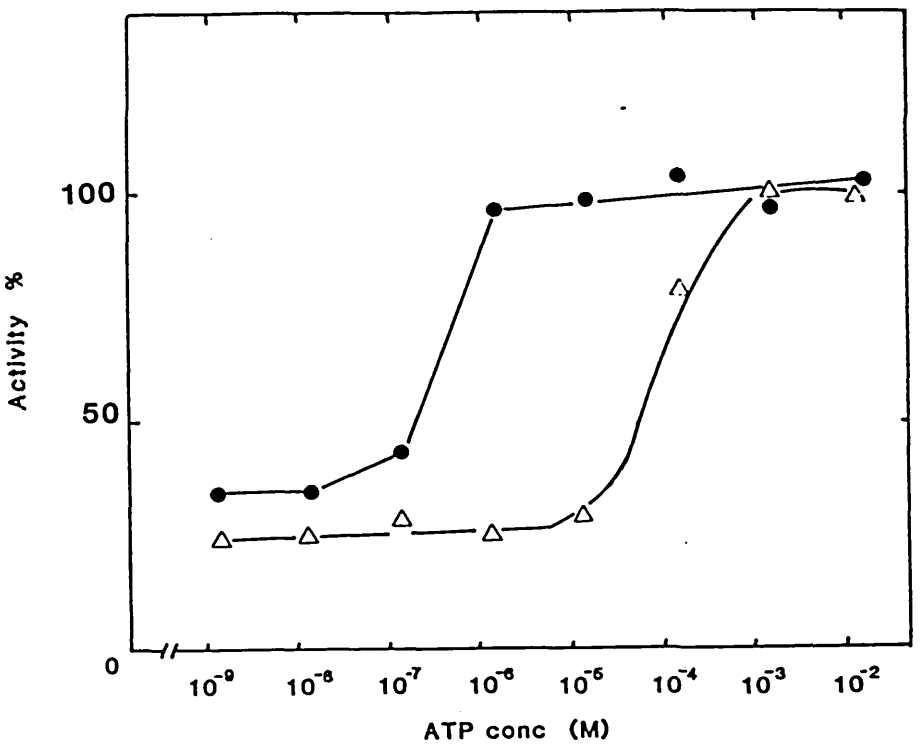


Fig. 3.9 Km for ATP of DNA ligase 1 (●) and DNA ligase II (Δ)
The enzymes were incubated with poly(dA)·oligo(dT) substrate in ligase buffer containing varying concentrations of ATP for 1h at 16°C.

carried out in the presence of 17.5% PEG 6000 (Fig. 3.10) and at 37°C. Under these conditions DNA ligase I was able to join the DNA fragments (lane 4) whereas DNA ligase II (lane 6) did not exhibit any detectable activity. Preincubation of DNA ligase I with antibodies against this enzyme inhibited its activity (lane 5), but had no effect on T_4 DNA ligase (lane 2 and 3). The activity of DNA ligase I with this substrate was proportional to enzyme concentration up to 1.5 x 10^{-6} units and time up to 60min (Fig. 3.11).

Several other parameters were investigated in attempts to detect blunt-end ligation activity with DNA ligase II. However, neither increasing the time of reaction whilst decreasing the temperature from 37°C to 16°C, nor a five fold increase in the amount of enzyme added revealed any activity. The reaction mixture was also supplemented with 50-200mM KCl. DNA ligase II still failed to exhibit any activity and high concentrations of this salt partially inhibited DNA ligase I.

The dependence of the blunt end joining reaction on PEG was determined by varying the concentration of this compound in the reaction mixture (Fig. 3.12). The quantity of blunt-end joining carried out by DNA ligase I increased with PEG concentration up to 17.5%, where maximum joining activity was observed. The activity decreases slightly in the presence of 22.5% PEG and no DNA ligase I activity was observed in the absence of PEG after 1h at 37°C. However, if the reaction was incubated for 72h at 16°C approximately 30% of the DNA was converted into the high molecular weight ligation product. Therefore, the rate of joining of blunt

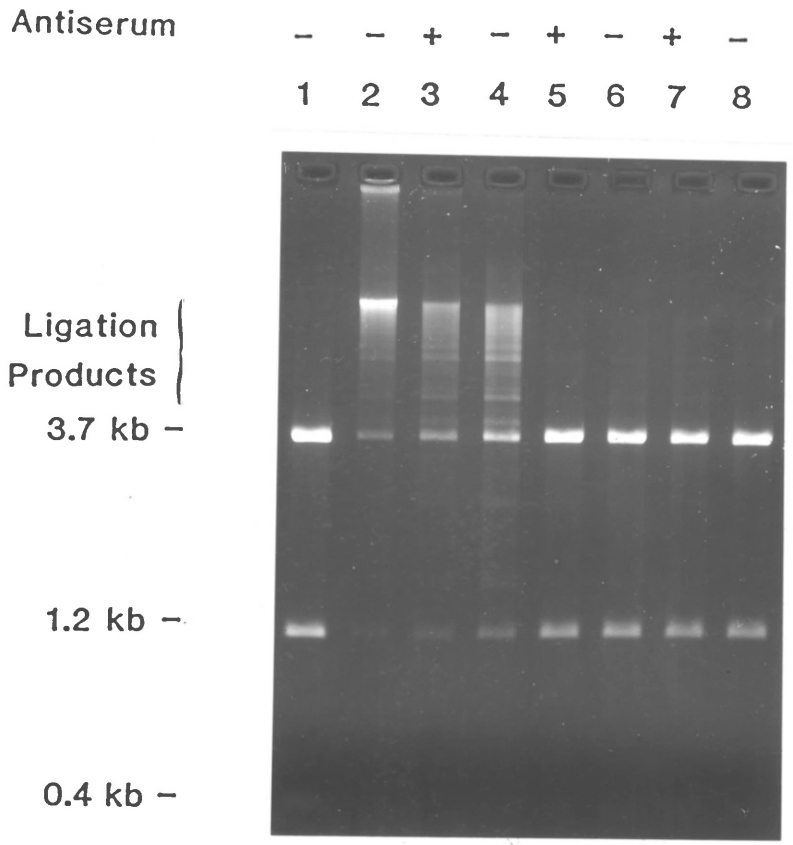


Fig. 3.10 **Blunt end joining by DNA ligases**
The enzymes were assayed for their ability to join HpaI fragments of ϕX174 replicative form DNA in the presence of 17.5% polyethylene glycol 6000. Reaction products were characterized by agarose gel electrophoresis. Lane 1 and 8, no enzyme added; Lanes 2 and 3, T4 DNA ligase; Lanes 4 and 5, mammalian ligase I; Lanes 6 and 7, mammalian ligase II. Antibody to ligase I was added as indicated.

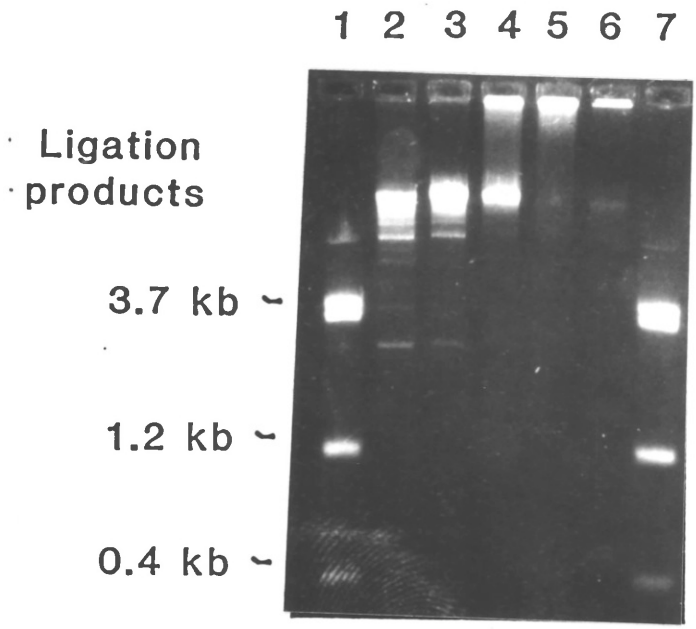


Fig. 3.11 Blunt end joining of DNA by varying concentrations of mammalian DNA ligase I. Reactions were performed in the presence of 17.5% (w/v) PEG 6000. Lanes 1 and 7 no enzyme; Lanes 2-6 0.02, 0.04, 0.06, 0.08 and 0.10μg of ligase I respectively.

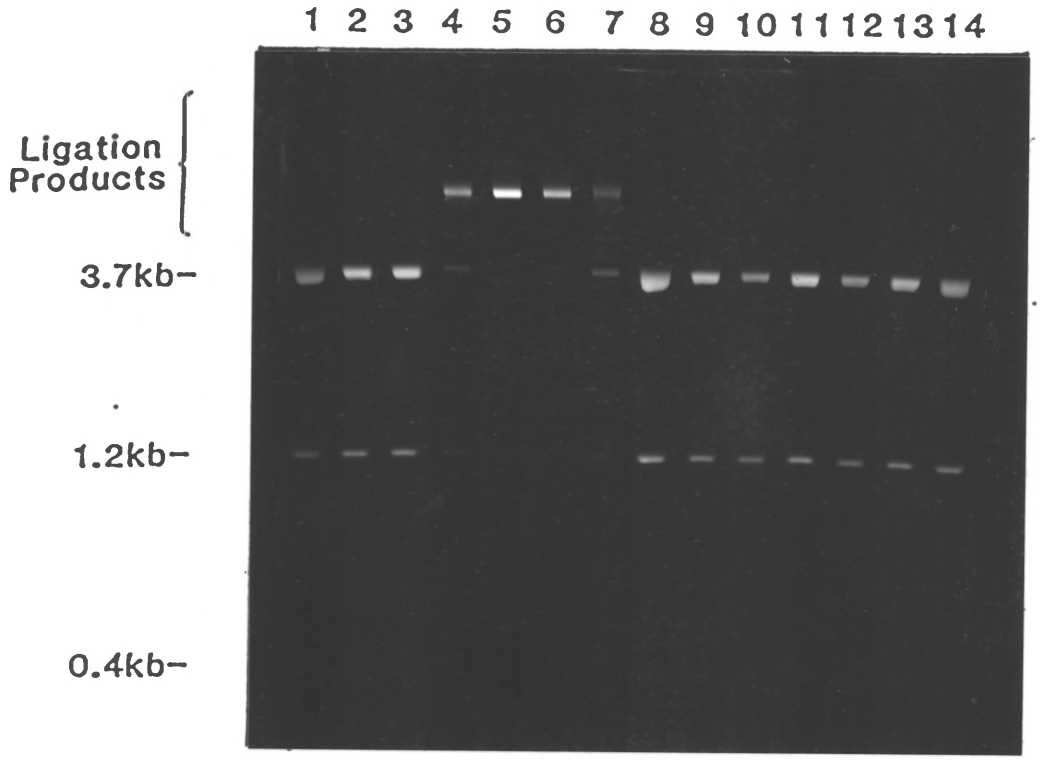


Fig. 3.12 Blunt-end joining by DNA ligases at different concentrations of polyethylene glycol 6000
Lanes 2-7, mammalian ligase I and 0, 7.5, 12.5, 17.5, 20, 22.5% of polyethylene glycol respectively; Lanes 9-14, mammalian ligase II and 0, 7.5, 12.5, 17.5, 20 and 22.5% of polyethylene glycol respectively. Lanes 1 and 8 do not contain enzyme.

end cut DNA by DNA ligase I in the absence of PEG occurs at a 25-30 times reduced rate. No detectable blunt-end joining activity was observed by DNA ligase II with any PEG concentration (Lanes 9-14).

It is concluded that in reactions containing PEG, DNA ligase I but not DNA ligase II can join blunt-ended DNA molecules.

3.3 DNA ligase I and II activities during different phases of cell growth

There are several lines of evidence which indicate that only one of the two enzymes may be required for essential DNA replication events. In rapidly proliferating mammalian cells DNA ligase I is the major species, comprising up to 90% of the total activity. The ratio of DNA ligase I to DNA ligase II is, however, dependent upon the tissue type. Hence, the ratios of ligase I to ligase II in a variety of tissues are: calf thymus 12:1; calf spleen 2:1; calf liver 1.5:1 and human placenta 1.4:1 (Soderhall & Lindahl, 1975). During rat liver regeneration DNA ligase I activity increases between 4 and 15 fold, whilst the levels of DNA ligase II remain constant (Soderhall, 1976; Chan & Becker, 1985). These data indicate a correlation between the rate of proliferation and DNA ligase I activity.

To investigate this further, cultured human cells from a Burkitts lymphoma cell line, Raji, were harvested at two stages of growth; when the cells were in exponential at 7 x

10^5/ml, or stationary at 6 x 10^6/ml phases of growth. Cells were extracted and the two ligase activities separated by hydroxyapatite chromatography as described in section (2.62).

The DNA ligase activity in the exponentially growing cells was ~2.0 times that of the stationary phase cells (Fig. 3.13). This difference in DNA ligase activity is much smaller than that observed in regenerating rat liver and probably reflects the fact that it is not possible to make this transformed cell line fully quiescent. The increase in DNA ligase I may be due to either enhanced expression of the DNA ligase I gene, post translational modification of DNA ligase I in the exponentially growing cells, or an accessory protein which could activate the endogenous DNA ligase. However, since other evidence (as discussed above) has indicated that DNA ligase I activity increases in rapidly proliferating tissues, an enhanced expression of DNA ligase I is the most likely explanation for these results. The DNA ligase II activity remained constant in the two growth phases. This demonstrates that whilst there may be relationship between growth and DNA ligase I, there is no similar relation for DNA ligase II and also suggests that DNA ligase II is probably not derived from DNA ligase I. If this were the case, the ligase II activity would have been expected to increase by the same extent as DNA ligase I.

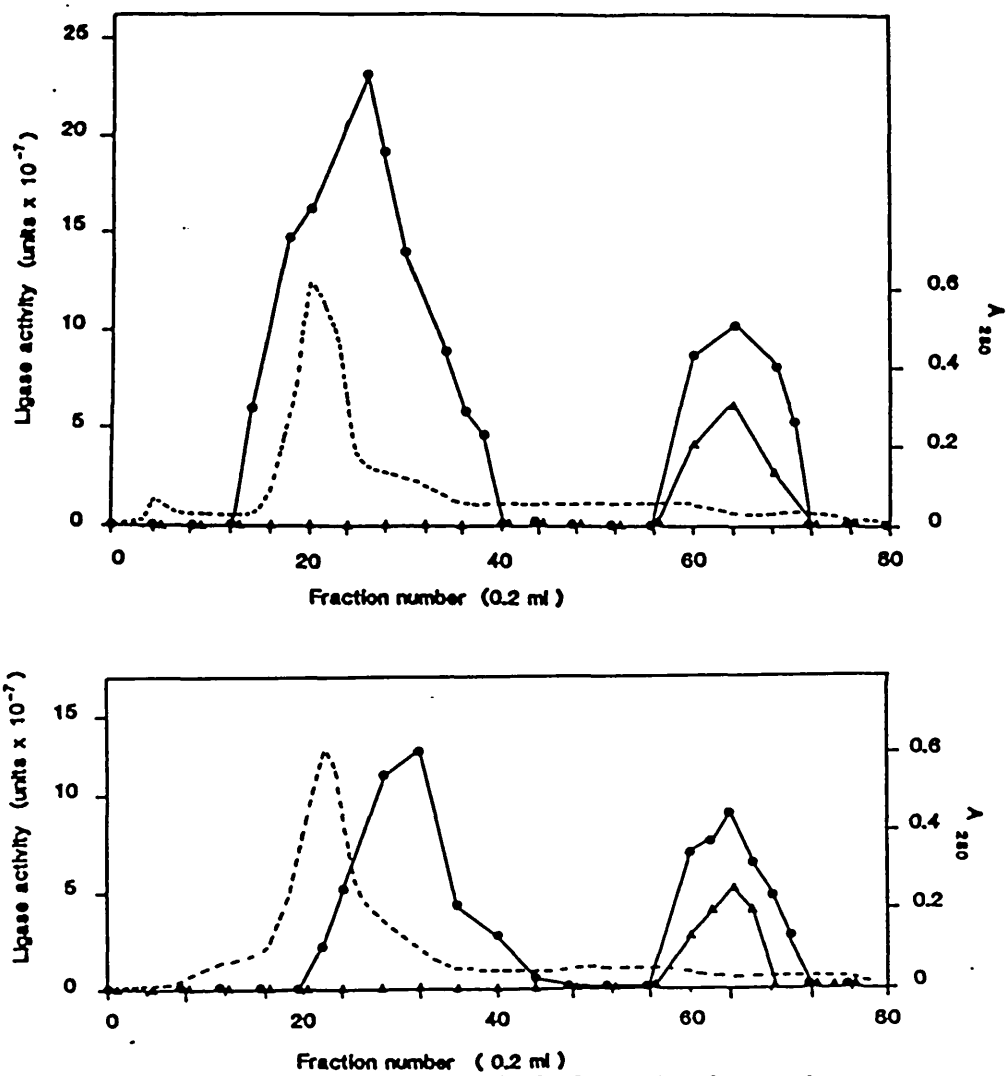


Fig. 3.13 DNA ligase I and II activities during a) exponential and b) stationary phases of growth
Cells were extracted and ligase activities separated by hydroxyapatite chromatography. The enzymes were assayed using poly(dA)·(oligo) dt(●), and poly(rA)·oligo (dT) (▲) substrates. Dashed line represents A_{280} (see section 2.62).

3.4 Investigation of DNA ligase activity in HeLa cells after incubation with dimethylsulphate

It has been proposed that DNA ligase II may be involved in DNA repair. Creissen and Shall (1982) postulated that DNA ligase II and poly ADP(ribosyl) transferase act together to repair damaged DNA. This is based on the observation that DNA ligase II activity was induced 2.5 fold after exposure of cells to the monofunctional alkylating agent dimethylsulphate (DMS). This apparent induction was prevented by 3-amino-benzamide an inhibitor of poly (ADP-ribose) biosynthesis (Durkacz et al., 1980).

The reaction sequence proposed to cause the increase in ligase II activity is as follows: Damage to cellular DNA stimulates the transfer of ADP-ribose by the enzyme poly (ADP-ribose) transferase to DNA ligase II forming a poly (ADP-ribose) modified protein. The ADP-ribose moiety enhances the DNA ligase activity, enabling sufficient enzyme activity to repair the DNA damage.

To investigate if this were a general phenomenon, these experiments were repeated. After treatment with dimethylsulphate, HeLa cells were extracted and the two enzymes, ligase I and II, separated on a FPLC Superose 12 column as described previously (Section 2.61). The DNA ligase I and II levels remained constant and no induction of either enzyme was observed (Fig. 3.14).

Therefore, whilst it is possible that DNA ligase II may have a function as a repair enzyme, these results indicate that it is not induced on this kind of DNA damage and the

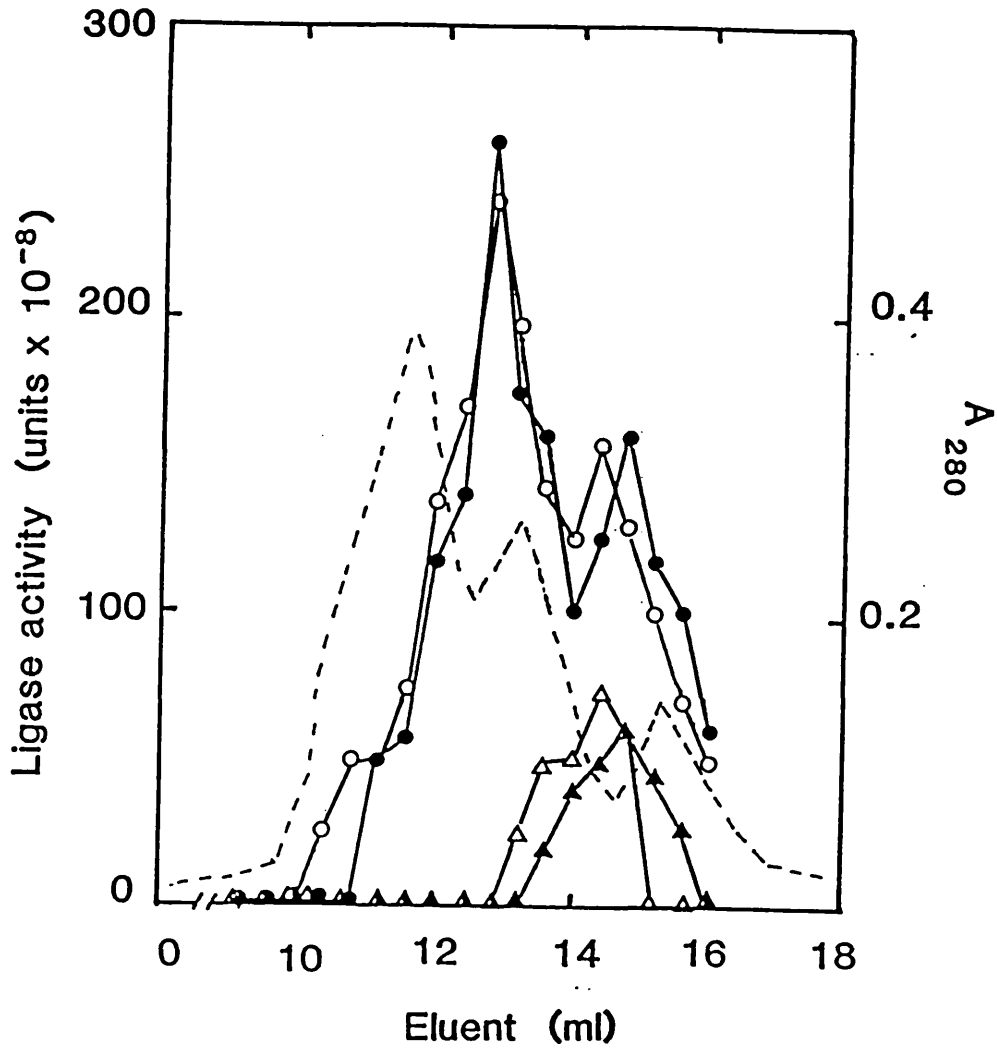


Fig. 3.14 DNA ligase activities in HeLa cells after pretreatment with DMS

Cells were extracted and DNA ligase activities separated by gel filtration on FPLC Superose 12. Circles show results with poly(dA)·oligo(dT) substrate and triangles poly(rA)·oligo(dT) substrate. Closed symbols denote results without DMS treatment, open symbols those with DMS treatment. Dashed line represents A_{280}.

repair pathway that it is associated with does not necessarily involve poly ADP-ribosyl transferase.

Summary

The data presented here indicate that mammalian cells, unlike bacteria and yeast, contain two DNA ligases. These enzymes exhibit different substrate specificities. Whilst these enzymes can join both 'nicked' DNA and poly(dA)·oligo(dT) substrates, DNA ligase I is the only enzyme capable of ligating blunt end cut DNA. In contrast, DNA ligase II is the only activity that can form phosphodiester bonds in a poly(rA)·oligo(dT) substrate and therefore exhibits a broader substrate specificity with regard to the complementary strand. DNA ligase I may be associated with DNA replication, but no physiological role has been shown for DNA ligase II. This latter enzyme may be involved in DNA repair.

Chapter 4

DNA ligase I deficiency in Bloom's syndrome

DNA ligase I deficiency in Bloom's syndrome

4.0 Introduction

This chapter presents data on the DNA ligase activities in several human inheritable syndromes, all of which exhibit a predisposition to certain forms of neoplasia. In addition, many of the cell lines derived from these inheritable syndromes are hypersensitive to a variety of DNA damaging agents at differing degrees of sensitivity, but a complete understanding of the underlying biochemical defects has not yet been achieved (Section 1, Table 2).

4.1 Results

4.1.1 Bloom's Syndrome Cell Lines with High Frequencies of Sister Chromatid Exchange and Defects in DNA Ligase I

The lymphoblastoid cell lines GM3403C and W674, and a fibroblast line GM8505 derived from three Bloom's syndrome patients of Ashkenazi Jewish origin, and one from a Mennonite, 1004, all exhibited high frequency of SCE. The exchange frequency was 60-80 per cell (Fig. 4.1), which are characteristic values in this disease.

The DNA ligase activities were determined in these cells and in a number of cell lines derived from other inheritable cancer-prone syndromes. The cell extracts were made as previously described (2.6), size fractionated

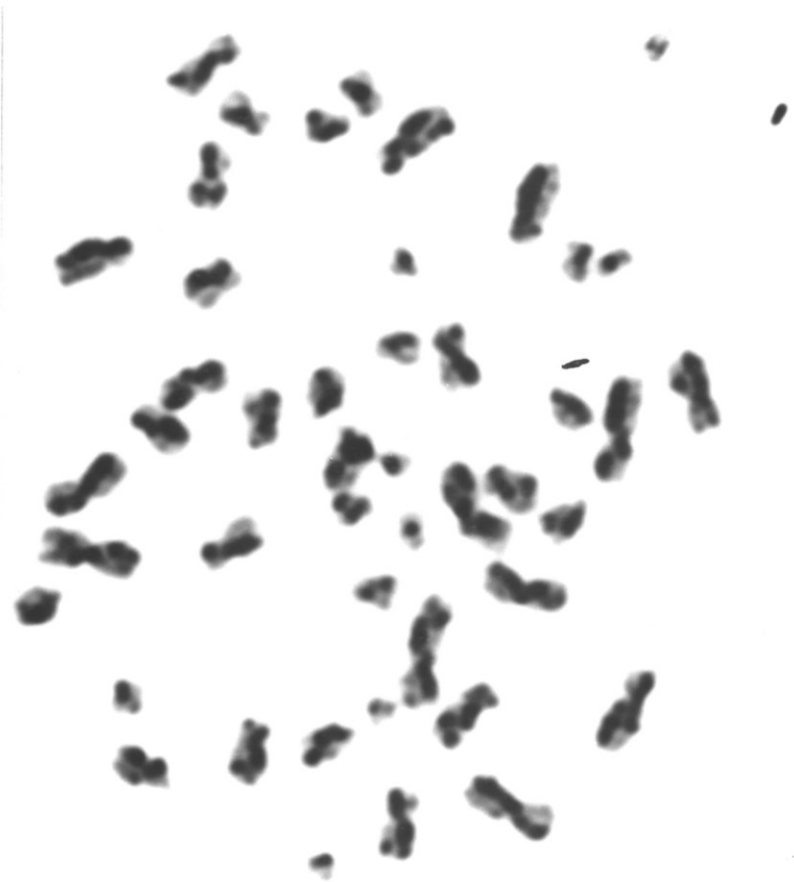


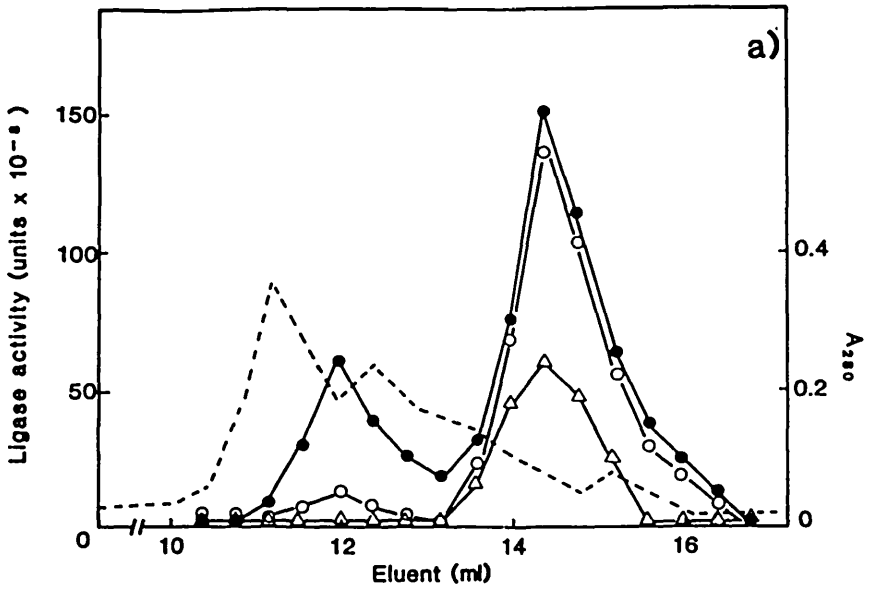
Fig. 4.1 Sister chromatid exchanges in Bloom's syndrome cell line W674

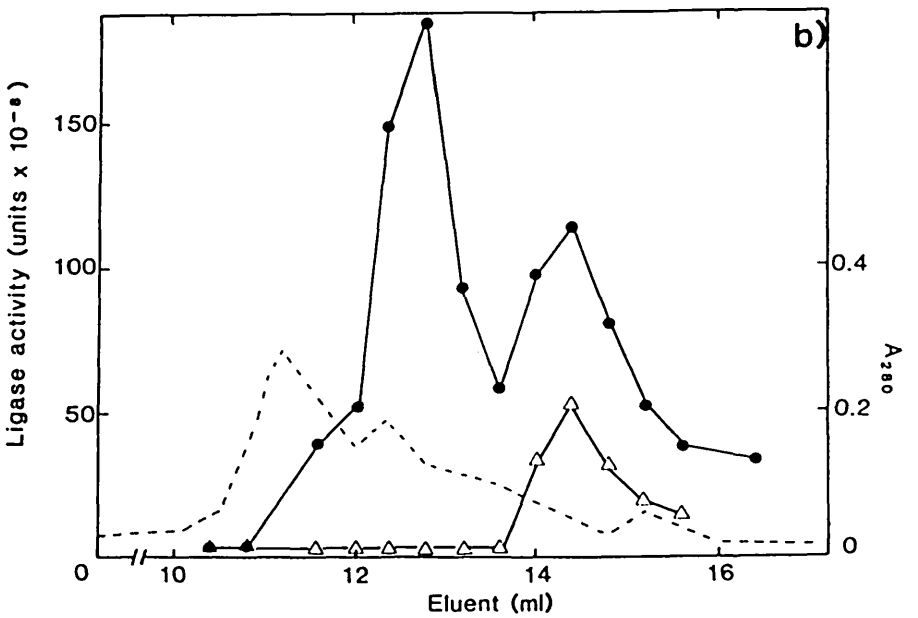
by liquid chromatography using a FPLC Superose 12 column and assayed for total ligase activity using the poly(dA)·oligo [5′-^{32}P](dT) substrate (2.35). In the control cell lines a large peak of DNA ligase I of relative molecular mass 200,000Da was observed. Under the conditions employed here, there was no evidence for the presence of dimers of DNA ligase I as reported by Chan & Becker (1985). The smaller peak of 80kDa represented DNA ligase II. This was verified using the poly(rA)·oligo [5′ ^{32}P](dT) assay. No other DNA ligase activity was detecting eluting after DNA ligase II. There have been a number of reports concerning the size heterogeneity of mammalian DNA ligase (Mezzina et al., 1984), and DNA ligase activity of a range of molecular weights 60, 70, 120, 150 and 200 has been observed. Under the extraction conditions used here only two peaks of DNA ligase activity were found. The apparent size heterogenity could be due to proteolytic cleavage of DNA ligase I.

The ratio of DNA ligase I activity relative to DNA ligase II was constant in all control cell lines, small differences were due to experimental variation. Therefore, in cell extracts from Werner's syndrome and Raji, the ratio of ligase I: ligase II activity was 1.7:1, whereas in extracts from ataxia telangiectasia and Cockayne's syndrome cells the ratios were 1.4:1 and 1.3:1 respectively (Figs. 4.2, 4.3, 4.4, 4.6 and 4.15). However, in the four cell lines GM8505, GM3404C, W674 and 1004 the ratio of DNA ligase I: DNA ligase II activity was

Fig. 4.2 **Size fractionation of DNA ligase activities in Bloom's syndrome cell line GM3403 (a) and xeroderma pigmentosum group A cell line GM2250(b)**

FPLC Superose-12 column profiles of polymin-P treated cell extracts are shown. Closed circles show results with the standard DNA ligase assay (Section 2.351) and open triangles the DNA·RNA hybrid assay specific for DNA ligase (II). Open circles (a) depict the standard DNA ligase assay in the presence of antiserum directed against calf thymus DNA ligase I. Dashed line represents A_{280}.


a)
Ligase activity (units x 10⁻⁸)
150
100
50
0
0.4
0.2
0
A280
10
12
14
16
Eluent (ml)


b)
Ligase activity (units x 10⁻⁸)
150
100
50
0
0.4
0.2
0
A280
10
12
14
16
Eluent (ml)

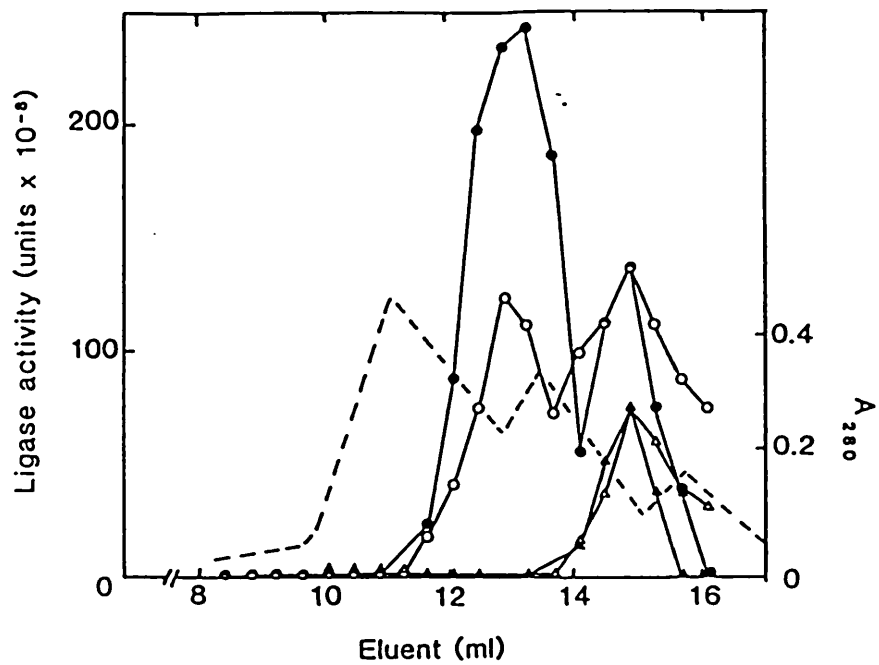


Fig. 4.3 DNA ligase activities in Bloom's syndrome cell line W674 and Werner's syndrome cell line AG3829

FPLC Superose-12 column profiles of Polymin-P treated cell extracts are shown. Circles show results with the standard DNA ligase assay, and triangles the DNA·RNA hybrid assay specific for DNA ligase II. Open symbols depict the results of the Bloom's syndrome cell line and closed symbols the 'control' cell line. Dashed line represents A_{280} of the Bloom's syndrome material, the A_{280} profile of the control cell line was similar.

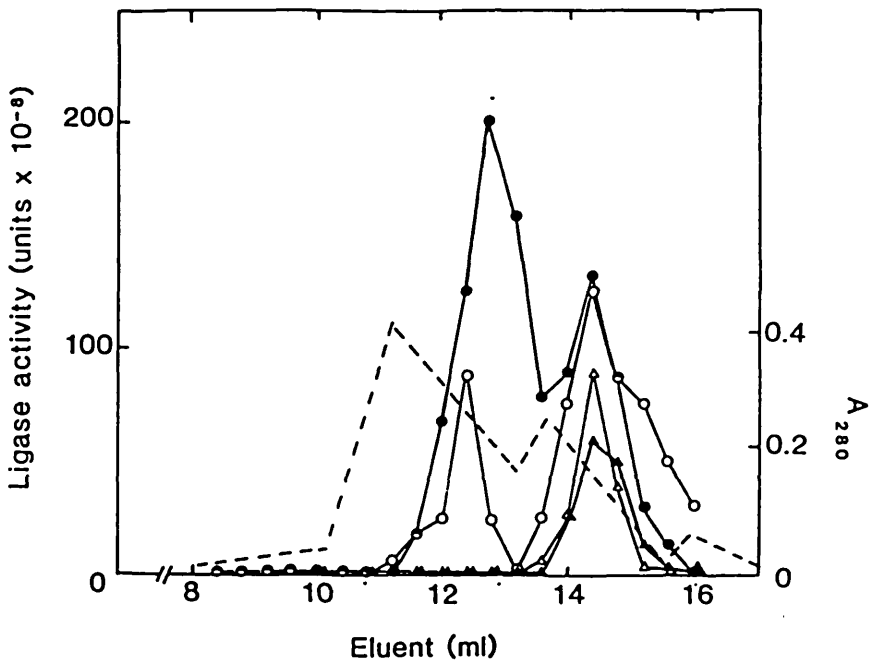


Fig. 4.4 DNA ligase activities in Bloom's syndrome cell line (GM8505) and HeLa
HPLC elution profiles and symbols are the same as in Fig. 4.3.

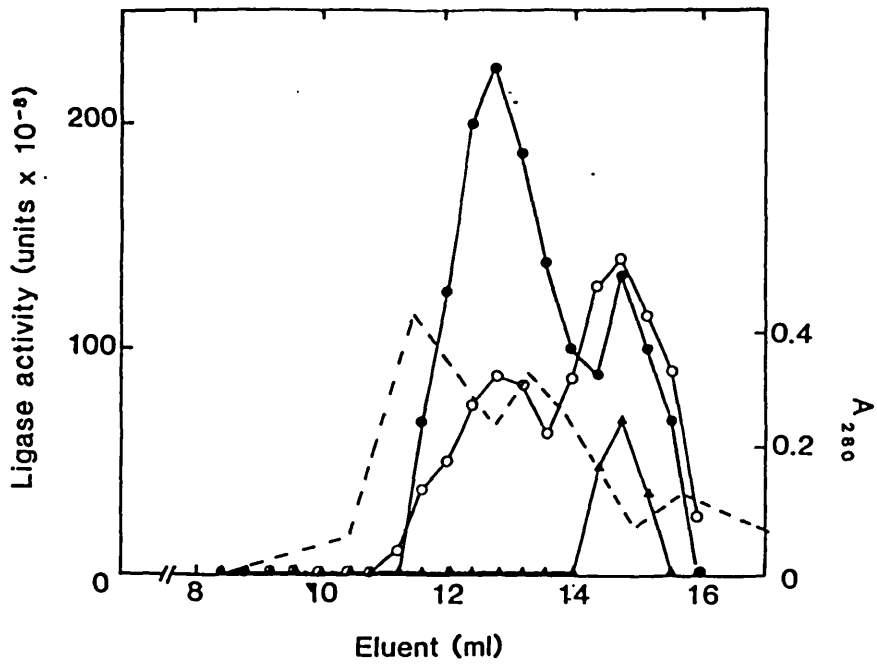


Fig. 4.5 DNA ligase activities in Bloom's syndrome cell line, 1004, and xeroderma pigmentosum variant, GM2249

FPLC elution profiles and symbols are the same as in Fig. 4.3.

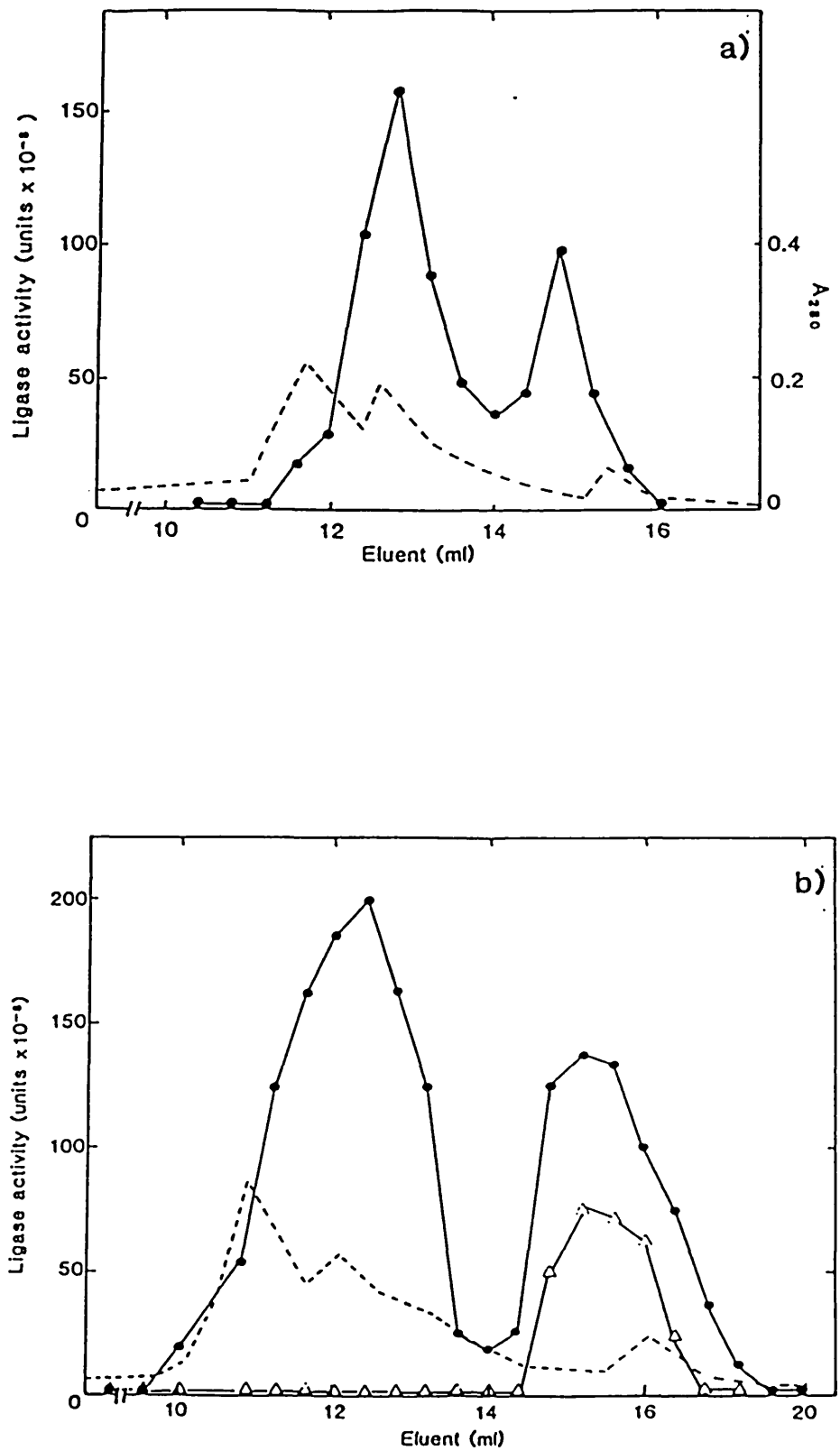


Fig. 4.6 DNA ligase activities in ataxia telangiectasia cell line, a), and Raji, b) FPLC elution profiles and symbols are the same as in Fig. 4.2.

lower. This reduction was due to a low level of DNA I activity in these cell lines (Figs. 4.2, 4.3, 4.4 and 4.5 respectively). The residual activity which eluted at the expected position of DNA ligase I in the GM3403C line was inhibited 80% by rabbit antibodies against DNA ligase I (Soderhall & Lindahl, 1976), Fig. 4.2. This confirms that the high M_r peak of DNA ligase represented a small amount of active DNA ligase I.

4.1.2 Thermolability of DNA ligase I

The reduction of the DNA ligase I activity in the Bloom's syndrome lines could be due to a regulatory mutation or to a structural defect in the enzyme itself. To distinguish between these two possibilities, the heat lability of the partially purified size fractionated DNA ligase I from the Bloom's syndrome lines, GM8505, GM3403C, W674 and 1004 was determined. The DNA ligase I from control cells had a half life of 6min at 50°C which is comparable to the value obtained for calf thymus DNA ligase I (Soderhall and Lindahl, 1975). The DNA ligase I from all four Bloom's syndrome cell lines was twice as heat labile, with a half-life of 3min at 50°C (Figs. 4.7, 4.8). The DNA ligase II from these cells showed the same heat lability as that from control cells with 50% inactivation occurring at 42°C.

There are many examples where heat lability of an enzyme indicates an alteration in its secondary structure. It is likely, therefore, that the reduction of the DNA

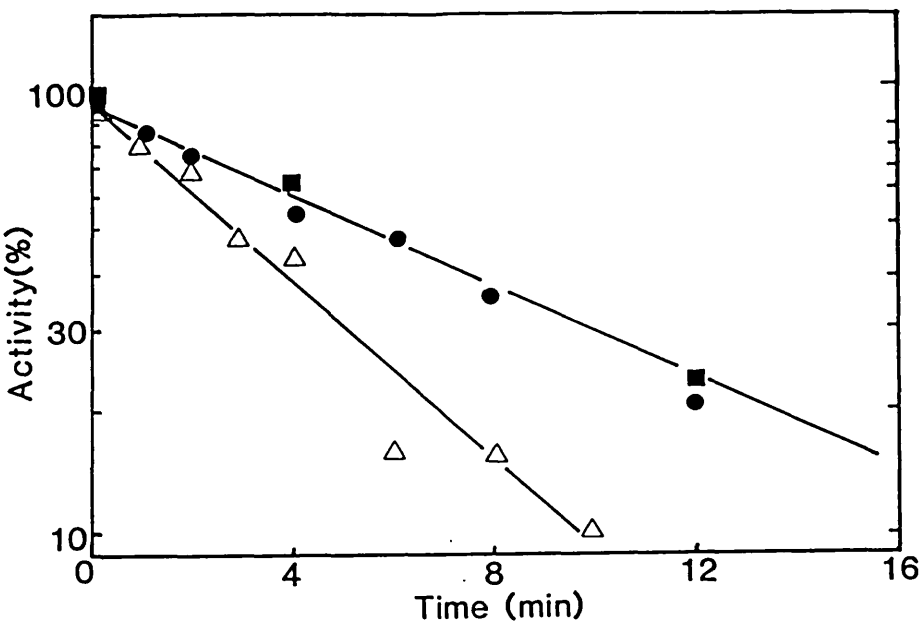


Fig. 4.7 Heat lability of DNA ligase I in Bloom's syndrome

Ligase I peak fractions from FPLC were incubated at 50°C and aliquots removed and assayed as described in (2.351). The open triangles indicate material from Bloom's syndrome line GM3403, closed circles, Raji and closed squares, xeroderma pigmentosum group A.

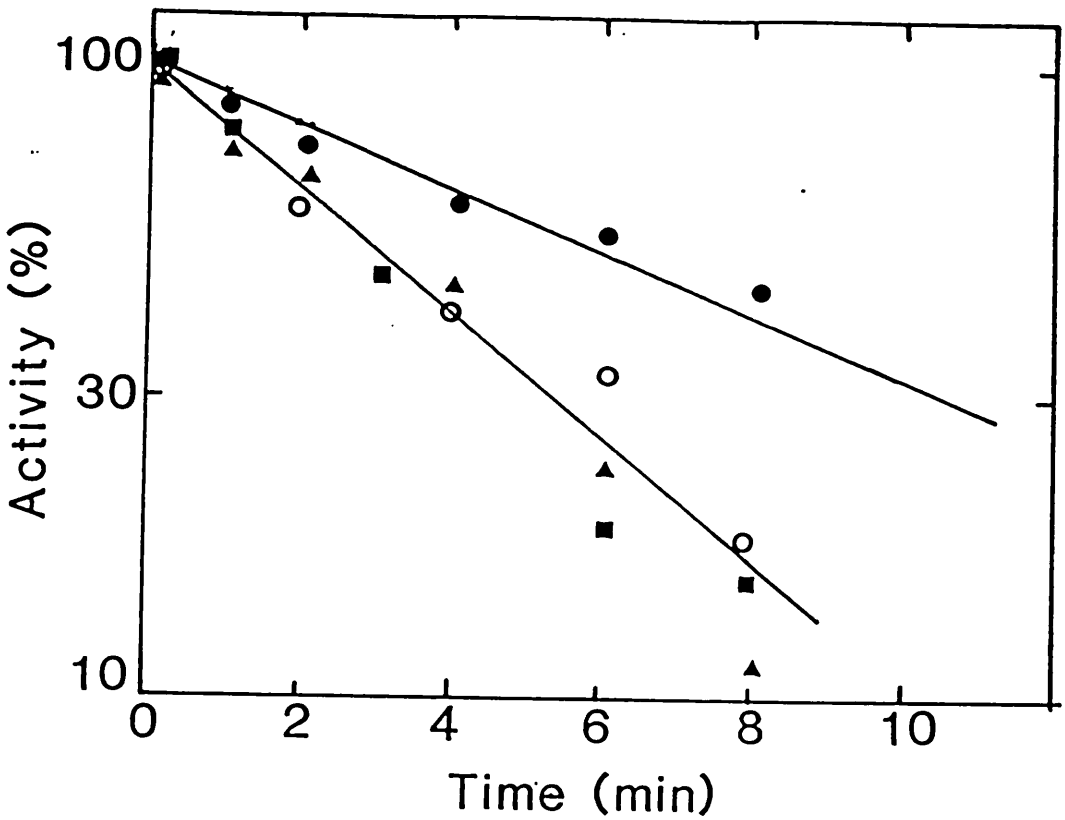


Fig. 4.8 **Heat lability of DNA ligase I from Bloom's syndrome**

DNA ligase I peak fractions from FPLC were incubated at 50°C and aliquots removed at predetermined time intervals and assayed as described (Section 2.351). The symbols indicate material from Bloom's syndrome line W67-4 (o), Mennoite Bloom's syndrome line 1004 (▲), Bloom's syndrome line GM8505 (■), and control line GM1953 (●).

ligase I activity in Bloom's syndrome cells is due to a structural alteration in the enzyme.

4.1.3 Bloom's Syndrome Cell Lines with Reduced Sister Chromatid Exchange and Defects in DNA Ligase I

In addition to the Bloom's syndrome cell lines with high frequencies of SCE, another class was observed which had a lower but still elevated level of SCE of 8-12 per cell, a two- to three-fold increase (Fig. 4.9). In this group are two cell lines derived from patients clinically diagnosed as Bloom's syndrome, AA87-5-1 and D86-1-2.

These cell lines also have a reduced DNA ligase I activity, containing 30% of the level of enzyme activity found in control cells (Figs. 4.10, 4.11). Therefore, there is no direct correlation between the SCE frequency and a reduction in DNA ligase I activity. The parental heterozygote cell line AA87-4, contained a higher level of ligase activity than the Bloom's syndrome material, but still exhibited rather a low ratio of DNA ligase I: DNA ligase II of 1:1.4. However, due to variation between experiments the DNA ligase profile from this heterozygote cell line cannot be distinguished from the Cockayne's syndrome cell line, which also has a low ratio of DNA ligase I: DNA ligase II. Furthermore, the difference between the DNA ligase I activity in the xeroderma pigmentosum variant line and the Friedreich's ataxia line is as large as the difference between the latter and the AA87-4 line.

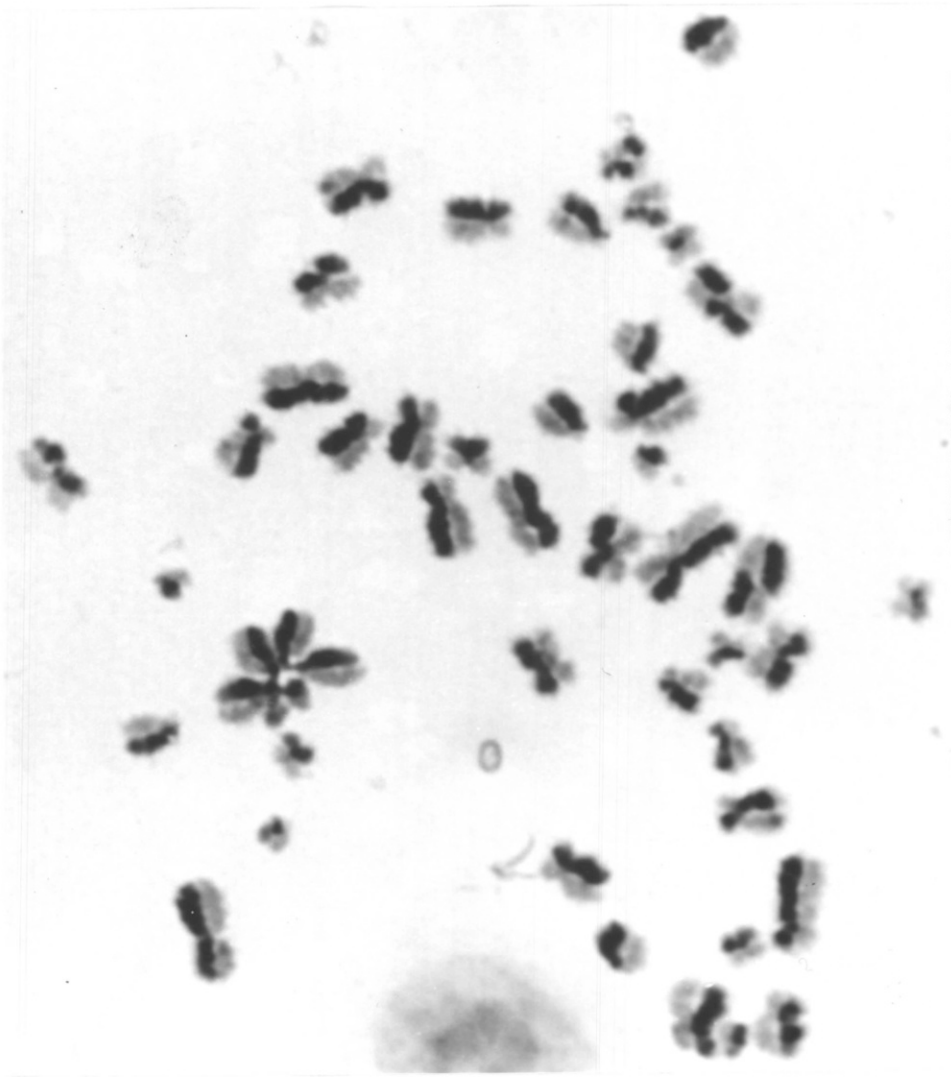


Fig. 4.9 Sister chromatid exchange in Bloom's syndrome cell line D86-1-2

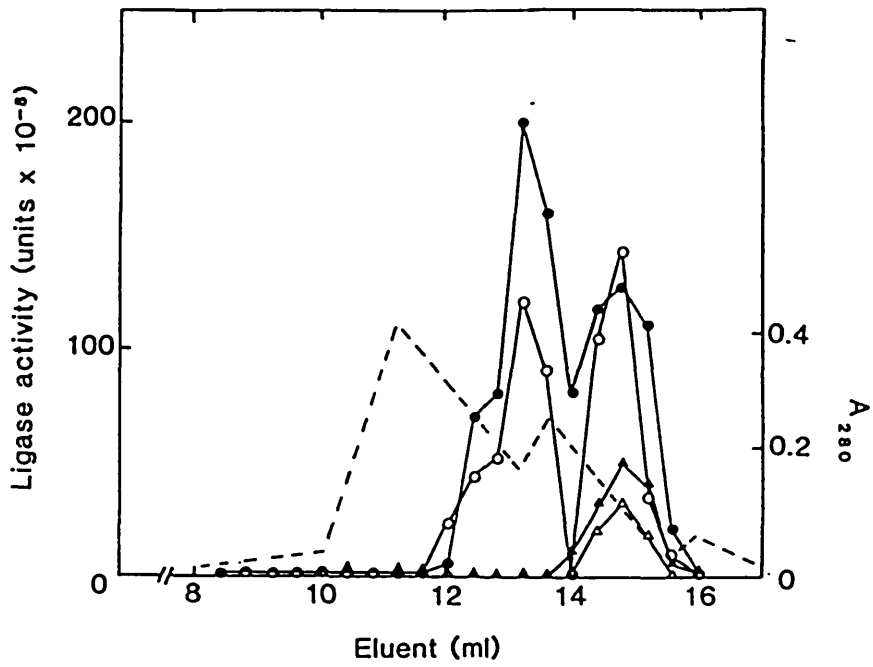


Fig. 4.10 DNA ligase activities in Bloom's syndrome cell line with low sister chromatid exchange D86-1-2, and the Friedreich's ataxia line, PS FPLC elution profiles and symbols are the same as in Fig. 4.3.

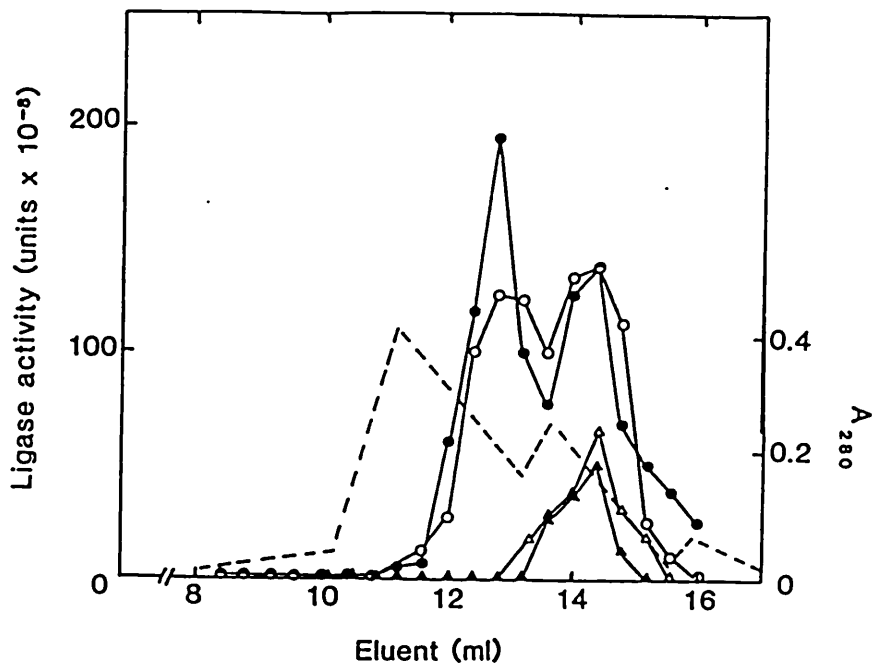


Fig. 4.11 DNA ligase activities in Bloom's syndrome cell line with low sister chromatid exchange AA87-5-1, and the heterozygote Bloom's syndrome cell line, A87-4
FPLC elution profiles and symbols are the same as in Fig. 4.3.

4.1.4 Heat Lability of DNA Ligase I

DNA ligase I partially purified from the two cell lines D86-1-2 and AA87-5-1 exhibited heat labilities similar to those found in the cell lines with high SCE frequencies, W674, GM3403, 1004, GM8505. This again indicates that there is no direct correlation between a defective DNA ligase I activity and the SCE frequency in these cells. The DNA ligase I activity in the D86-1-2 and AA87-5-1 cells also had a half life of three minutes at 50°C (Fig. 4.12). It would be expected that the heat lability of the heterozygote AA87-4, would be between that of the homozygous BS cells and control cells. A heterozygote cell line would be expected to contain 50% heat labile defective DNA ligase I and 50% that exhibits normal thermolability. However, because the experimental variation for the heat lability of the DNA ligase at each predetermined time point is approximately 10% it is not possible to distinguish between the heterozygote cell line and the control line, except perhaps by performing many repeated experiments.

4.1.5 A Revertant Bloom's Cell Line with no Defect in DNA Ligase I

Two cell lines established from an Ashkenazi Jewish individual patient show differences in their frequencies of SCE. The fibroblast cell line GM3498 has a high number of SCE, 60-80 per cell, whilst a lymphoblastoid cell line GM4408, has a normal SCE frequency. The DNA ligase

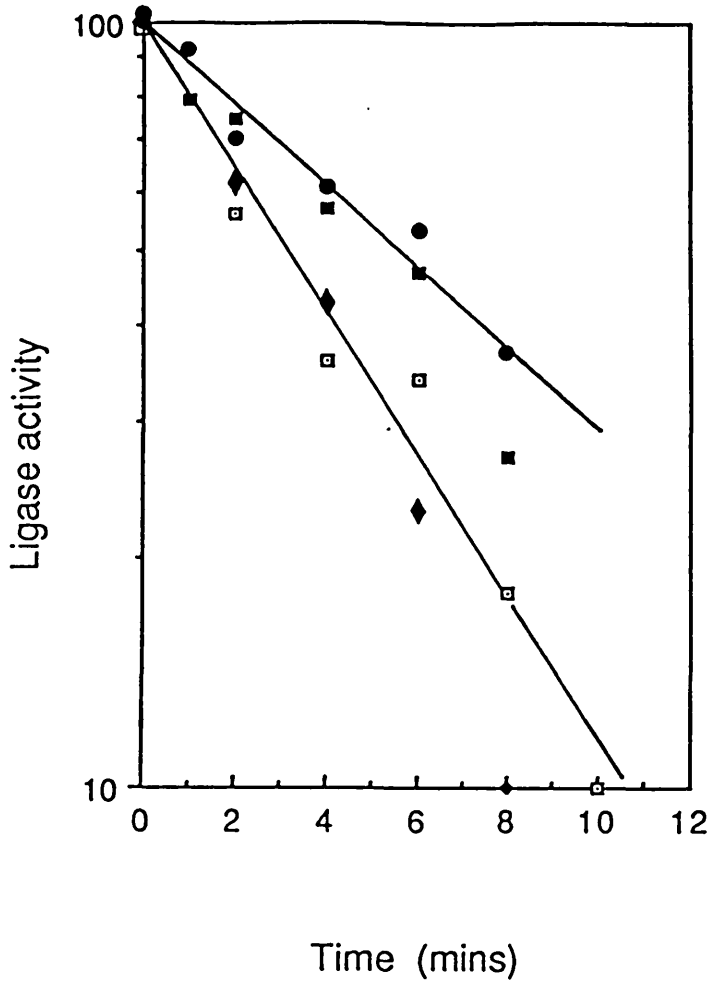


Fig. 4.12 **Heat lability of DNA ligase I from Bloom's syndrome**

Ligase I peak fractions from FPLC were incubated at 50°C and aliquots removed and assayed as described (2.). Symbols indicate material from Bloom's syndrome lines D86-1-2 (⧈), AA87-5-1 (♦), AA87-4 (■) and Raji (●).

activity in GM4408 was examined and the ratios of DNA ligase I: DNA ligase II were surprisingly found to be normal (Fig. 4.13).

To investigate if these cell lines were derived from the same patient, restriction fragment length polymorphisms and isoenzyme analysis were carried out by Dr. N. Spurr. Both these tests confirmed that the lines were from the same patient, suggesting that GM4408 may be a true revertant.

4.1.6 A Second Type of Structural Alteration in DNA Ligase I

Three cell lines have been examined which exhibit a different alteration in DNA ligase I. Two of the cell lines, 1032 and GM5289, were derived from Bloom's syndrome patients of French Canadian and Japanese origin, respectively. These both exhibited high frequencies of SCE.

The cell line 46BR (Webster et al., 1982) was derived from a patient, of Anglo-Saxon origin, who exhibited clinical characteristics similar to Bloom's syndrome. The patient showed immunodeficiency associated with dwarfism and dilated venous capillaries. The main difference between these cells and Bloom's syndrome cells can be seen in the frequencies of SCE per cell (46BR has a near normal frequency) (Teo et al., 1983b)

In each case when extracts from these cell lines were chromatographed, a proportion of the DNA ligase

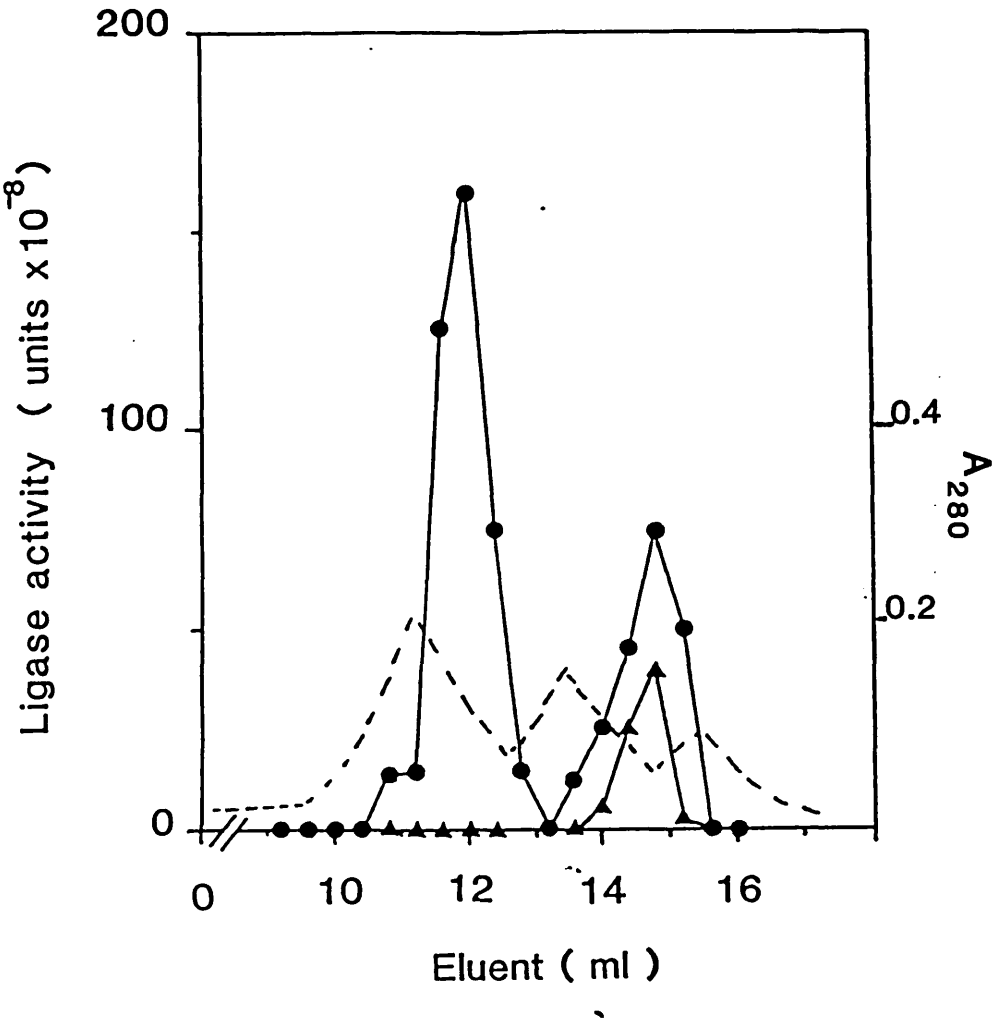


Fig. 4.13 DNA ligase activities in Bloom's syndrome cell line GM4408
FPLC elution profiles and symbols are as in Fig. 4.3.

activity eluted from the column at a position that corresponded to a molecular mass of 400kDa. The cell line 1032 was the most extreme case, with all the enzyme present in this form (Fig. 4.14). The results obtained with 46BR and GM5289 were very similar with between 40-60% of the ligase activity in the high molecular weight form (Fig. 4.15 and 4.16).

The high molecular weight form of DNA ligase I observed in these cell lines could be due to a large unprocessed active precursor of DNA ligase I, the DNA ligase I activity bound to another protein of the same molecular mass, or that the enzyme had dimerized.

When extracts of 1032 cells were treated with 1M NaCl, prior to chromatography, the DNA ligase activity converted to a form that eluted at the expected position of DNA ligase I (Fig. 4.14). These results suggest that in 1032 cells DNA ligase I is present in an active dimeric form, which can be dissociated by high salt concentrations.

To further test this hypothesis, extracts of 1032 cells were analysed by sucrose gradient centrifugation in the presence of different salt concentrations. Following sedimentation in the presence of 0.5M NaCl, the DNA ligase I from 1032 cells was observed as a very broad peak (Fig. 4.17). This was possibly due to a gradual dissociation of the dimeric form to the monomeric form. The addition of 1M NaCl to 1032 extracts prior to centrifugation caused a shift in the peak of ligase activity, which also became

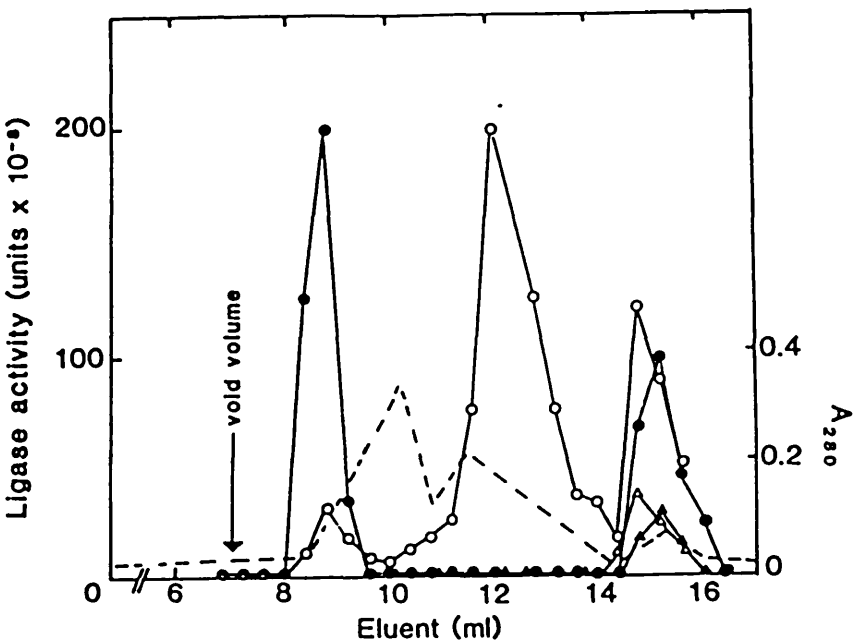


Fig. 4.14 DNA ligase activities in the Anglo-Saxon Bloom's syndrome cell line 1032, containing a dimeric form of ligase I

FPLC profiles as in Fig. 4.3 for closed symbols. The open symbols show the same experiment, but with incubation of the cell extract in 1M NaCl for 1h at 0°C prior to chromatography.

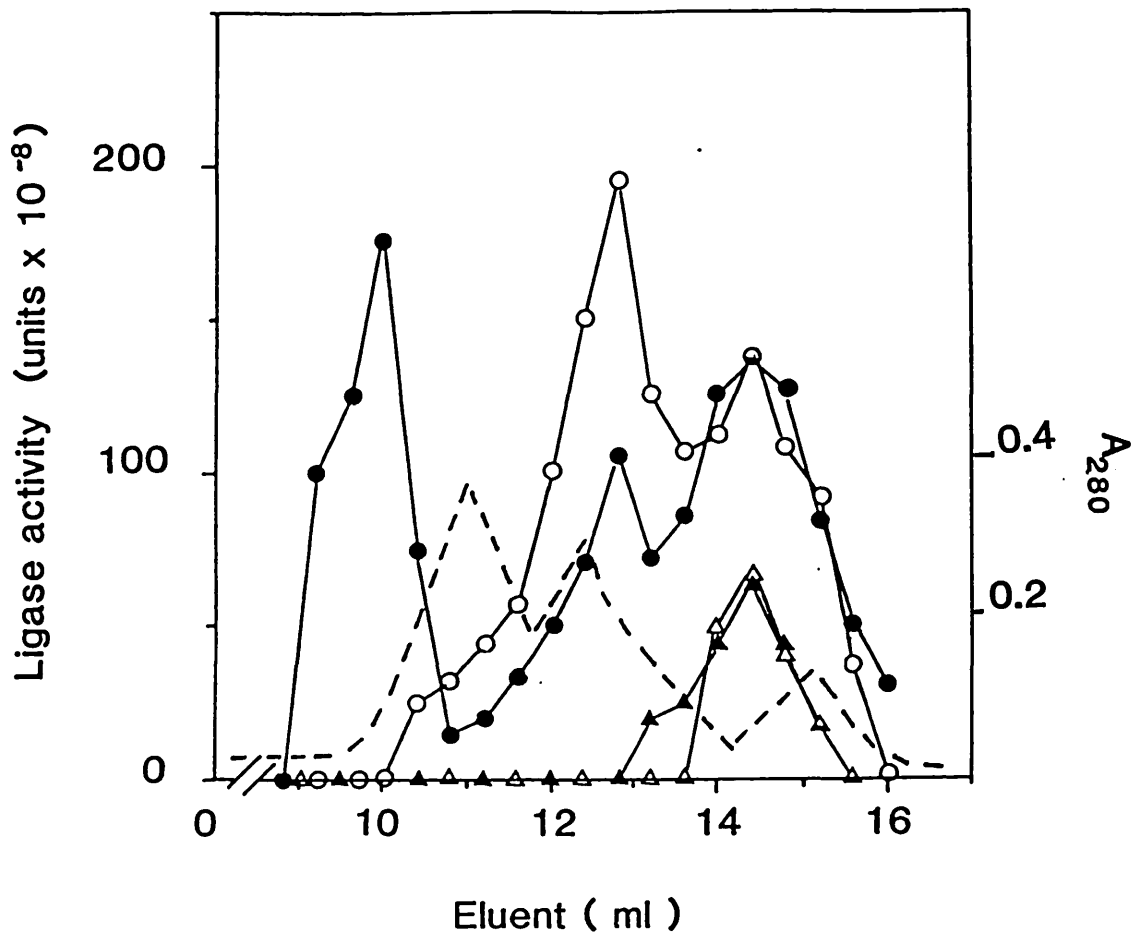


Fig. 4.15 DNA ligase activities in the cell line 46BR, containing a dimeric form of DNA ligase I and in Cockayne's syndrome cell line, GM1712. FPLC elution profiles and symbols are the same as in Fig. 4.3.

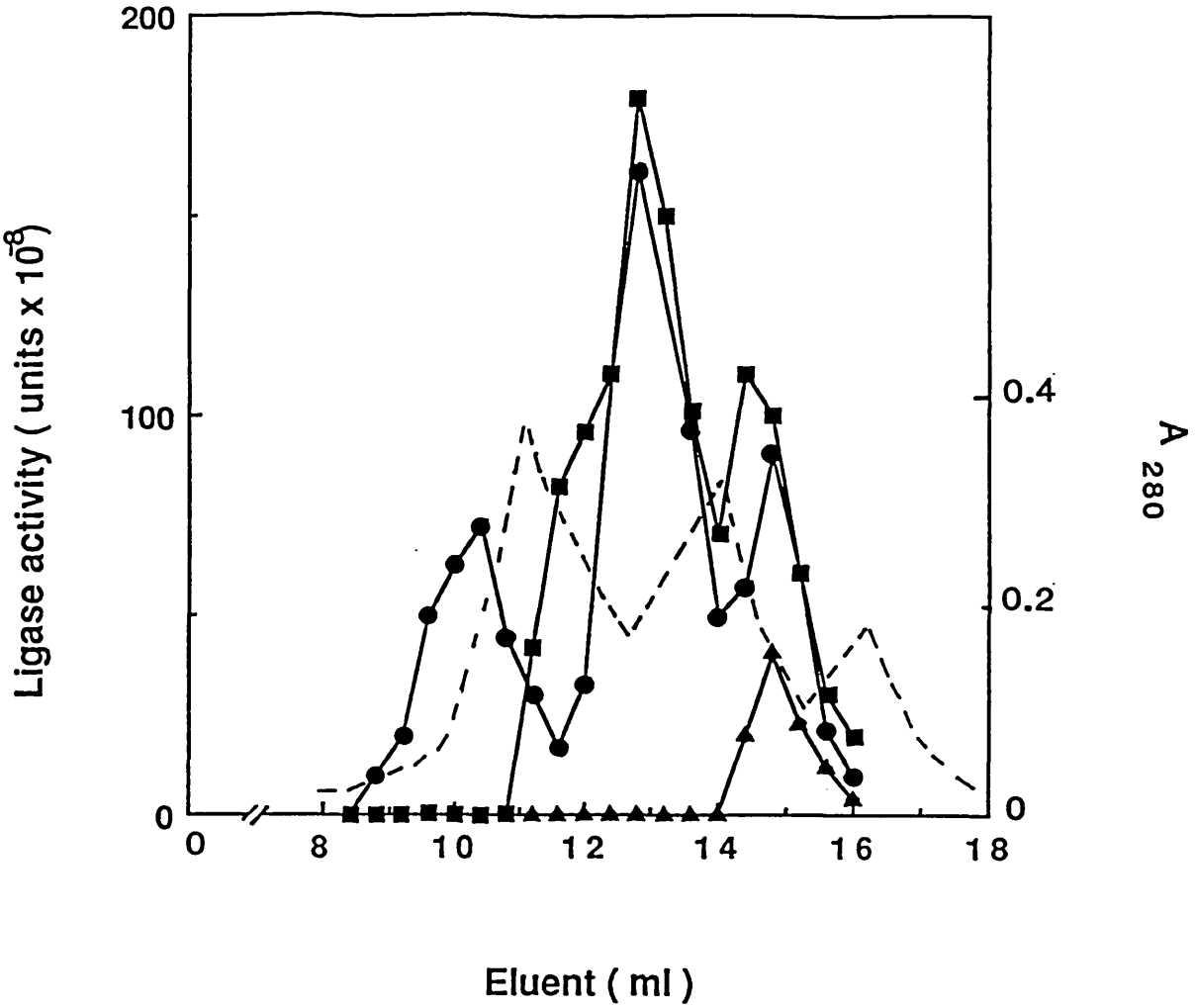


Fig. 4.16 DNA ligase activities in the Bloom's syndrome cell line GM5289A containing a dimeric form of DNA ligase I and in Fanconi's anaemia cell line GM4510.

much sharper and exhibited sedimentation properties similar to the DNA ligase I in extracts from xeroderma pigmentosum group A cells, i.e. the monomeric form. Heating the 1032 extracts to 40°C for 5min did not dissociate the high molecular weight form, but inactivated the second ligase peak, indicating that this activity could be attributed to DNA ligase II (Fig. 4.17).

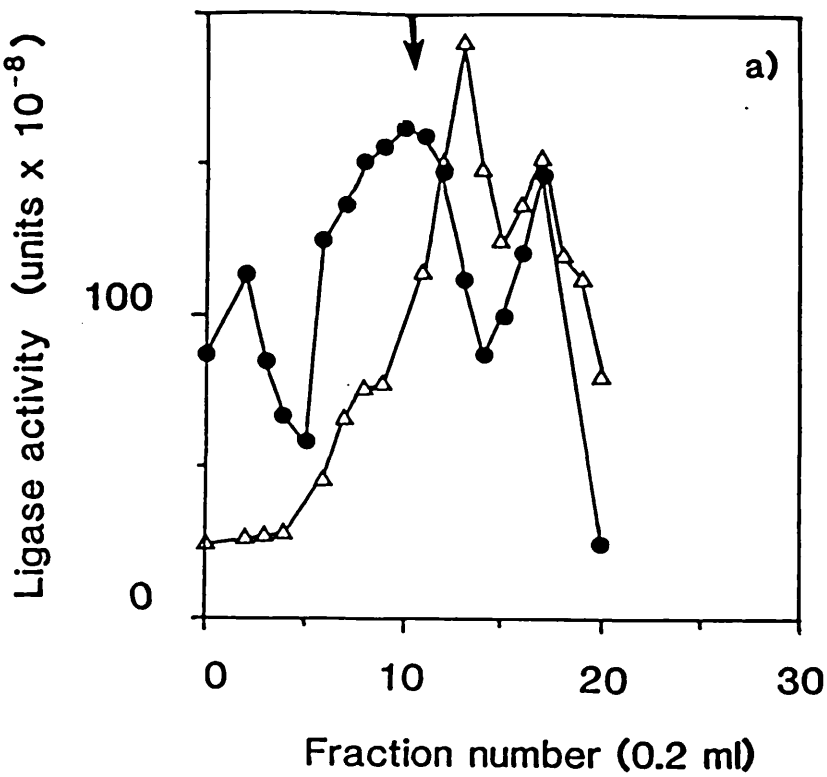
Sucrose gradient centrifugation at a NaCl concentration of 0.2M yielded three DNA ligase peaks in 1032 cells. These corresponded to the two forms of DNA ligase I, with the majority of the DNA ligase I activity present in the dimeric form (Fig. 4.18). The DNA ligase I activity in the Bloom's syndrome cell line W674 and xeroderma pigmentosum group A cells had sedimentation coefficients of 9S, similar to those obtained with DNA ligase I from calf thymus DNA (Soderhall & Lindahl, 1973(b)). The two DNA ligase I peaks observed in the 1032 cell extracts had sedimentation coefficients of 9S and 13S (Fig. 4.19). By combining Stokes radius data obtained by gel filtration with the sedimentation coefficients in the Svedberg equation (Siegel & Monty, 1966) molecular weights of approximately 200kDa and 400kDa, respectively, were estimated for the two forms of DNA ligase I.

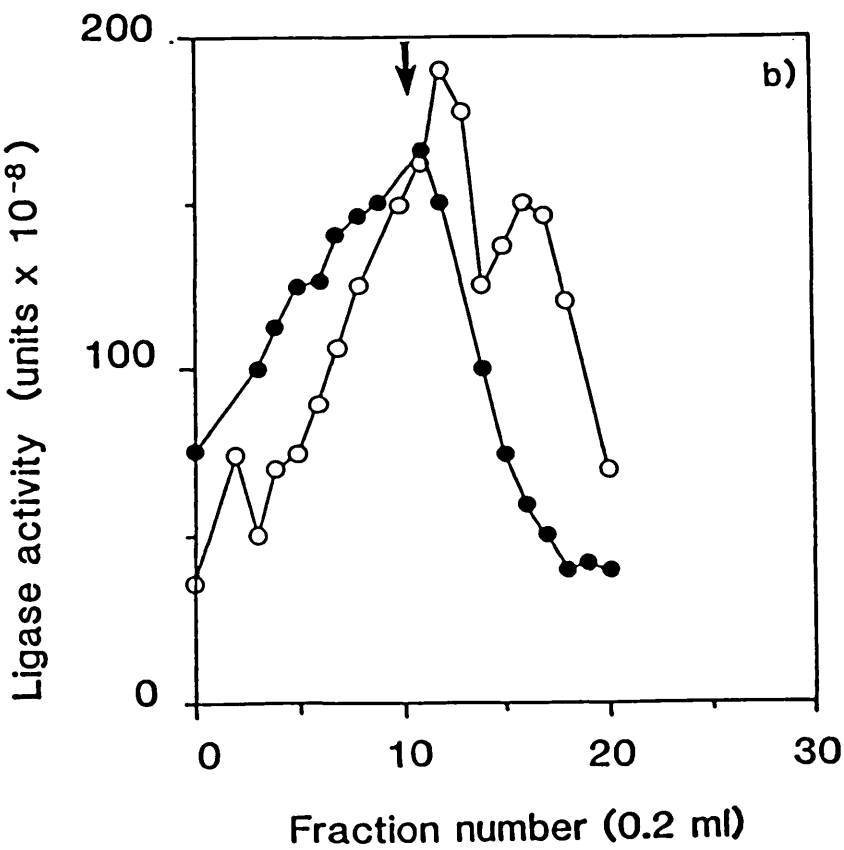
The dissociation of the DNA ligase I activity by high salt concentration indicates that the ~400kDa form of this enzyme is not due to a large precursor of DNA ligase I. Furthermore, the lower value obtained for the molecular weight of this protein by sucrose gradient

Fig. 4.17 Sucrose gradient centrifugation

(a) Cell extracts from the Bloom's syndrome cell line 1032 (●) and Xeroderma pigmentosum group A cell line GM2250 (△) were layered onto 5ml sucrose gradients (5-20%) containing 500mM NaCl 50mM Tris·HCl pH7.5, 100mM 2-mercaptoethanol and 1mM EDTA (Section 2.54). The arrow marks the position of catalase. Direction of sedimentation is towards the left.

(b) Extracts from the Bloom's syndrome cell line 1032, were treated with either 1M NaCl (o) or heated to 40°C for 5mins (●) prior to layering on to a 5ml sucrose gradient (5-20%).


a)
Ligase activity (units x 10^{-8})
100
0
0
10
20
30
Fraction number (0.2 ml)


b)
200
Ligase activity (units x 10^{-8})
100
0
0
10
20
30
Fraction number (0.2 ml)

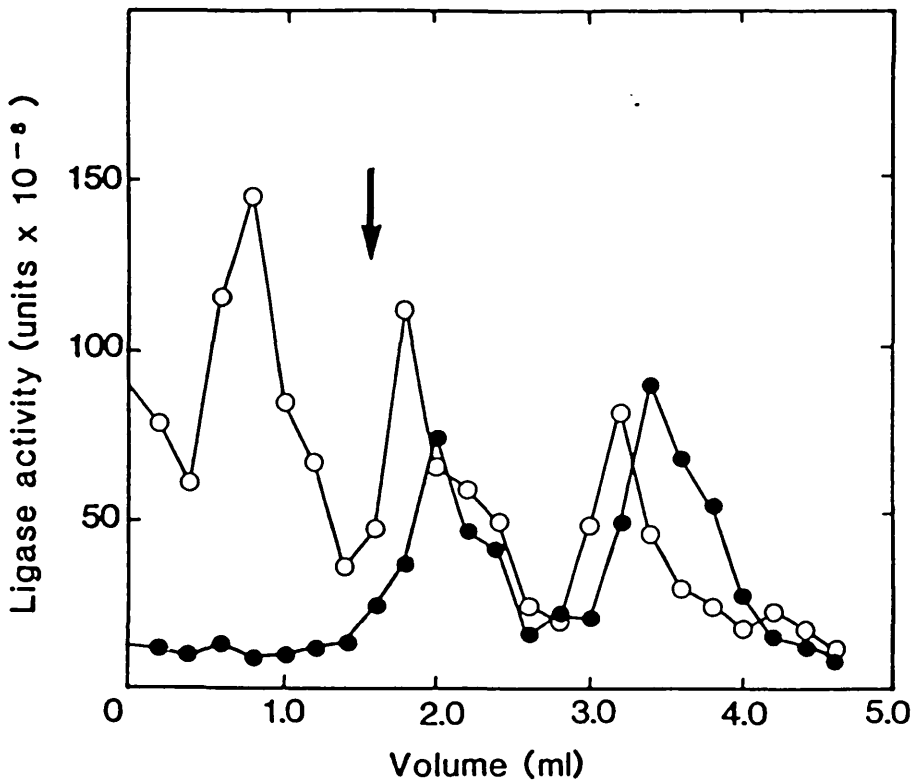


Fig. 4.18 Sucrose gradient centrifugation
Cell extracts from the Bloom's syndrome cell lines W1032 (o) and W674 (●) were layered on 5ml sucrose gradients (5-20%) containing buffer (2.54)with 200mM NaCl. The arrow marks the position of catalase which was used as an internal marker. Direction of sedimentation is towards the left.

centrifugation, when compared to gel filtration, suggests an asymmetric protein. It has been reported previously (Soderhall and Lindahl, 1973) that calf thymus DNA ligase I is an assymetric protein with a f/fo of 1.4. Therefore, the most likely explanation for the data obtained with extracts from the three cell lines 1032, 46BR and GM5289 is that the DNA ligase I activity is present in a dimeric form.

4.1.7 Heat Inactivation of DNA Ligase I

DNA ligase I from size fractionated extracts of the three cell lines, 1032, GM5289 and 46BR, were heated at 50°C. However, the DNA ligase activity in these cells did not appear to be any more heat labile than that of control cells (Fig. 4.20).

The type of mutation that gives rise to these defects in Bloom's syndrome cells is probably different from that observed in cell lines derived from patients of Askenazi jewish origin. The dual position of DNA ligase I on gel filtration columns would make it possible to screen for heterozygotes, which would be expected to have 50% of DNA ligase I in the monomeric form and 50% in the dimeric form in each of the two sizes.

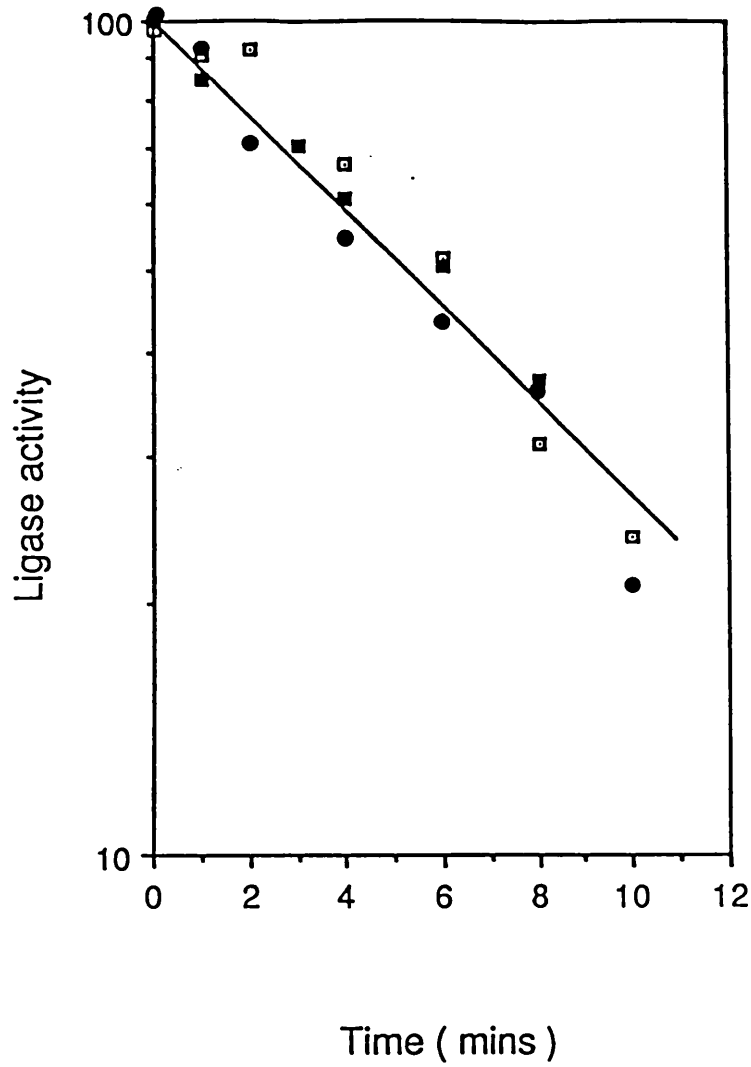


Fig. 4.20 Heat Inactivation of DNA ligase I from Bloom's syndrome cell lines containing a dimeric form of the enzyme

DNA ligase I from size fractionated extracts of 1032 (⊡), 46BR (■) and GM5289 (●) were incubated at 50°C and aliquots removed at predetermined time intervals and assayed for enzyme activity (section 2.351).

4.1.8 Experiments on DNA ligase I from Bloom's syndrome Cells

4.1.9 Purification of DNA ligase I from Bloom's syndrome

DNA ligase I from Bloom's syndrome cells GM3403C and from Raji cells were further purified (as described in section 2.63). Cell pellets from 3 litre cultures of each of the cell types were extracted as described previously (section 2.61). The extracts were fractionated by chromatography on AcA34 and DNA ligase I activity assayed. This type of analysis confirmed the data obtained using Superose 12, and indicated that there was less DNA ligase I activity in the Bloom's syndrome cell extracts than in the control cell extracts (Fig. 4.21), with a 70% reduction in the amount of enzyme present.

The DNA ligase I peaks from each cell type were separately pooled and loaded onto DNA cellulose columns (section 2.13, method described previously). In each case, the DNA ligase I activity bound to the column and could be eluted by the addition of NaCl (100mM), indicating no large alteration in the DNA binding affinity of the protein in Bloom's syndrome cells. The enzyme was purified approximately 20-fold by these two procedures.

Using this partially purified material blunt-end joining reaction were carried out, and in each case the DNA ligase I was able to ligate blunt ended DNA.

The enzyme from BS cells was observed to be as labile as before with a $t_{\frac{1}{2}}$ of 2.5min at 50°C. The DNA ligase I from

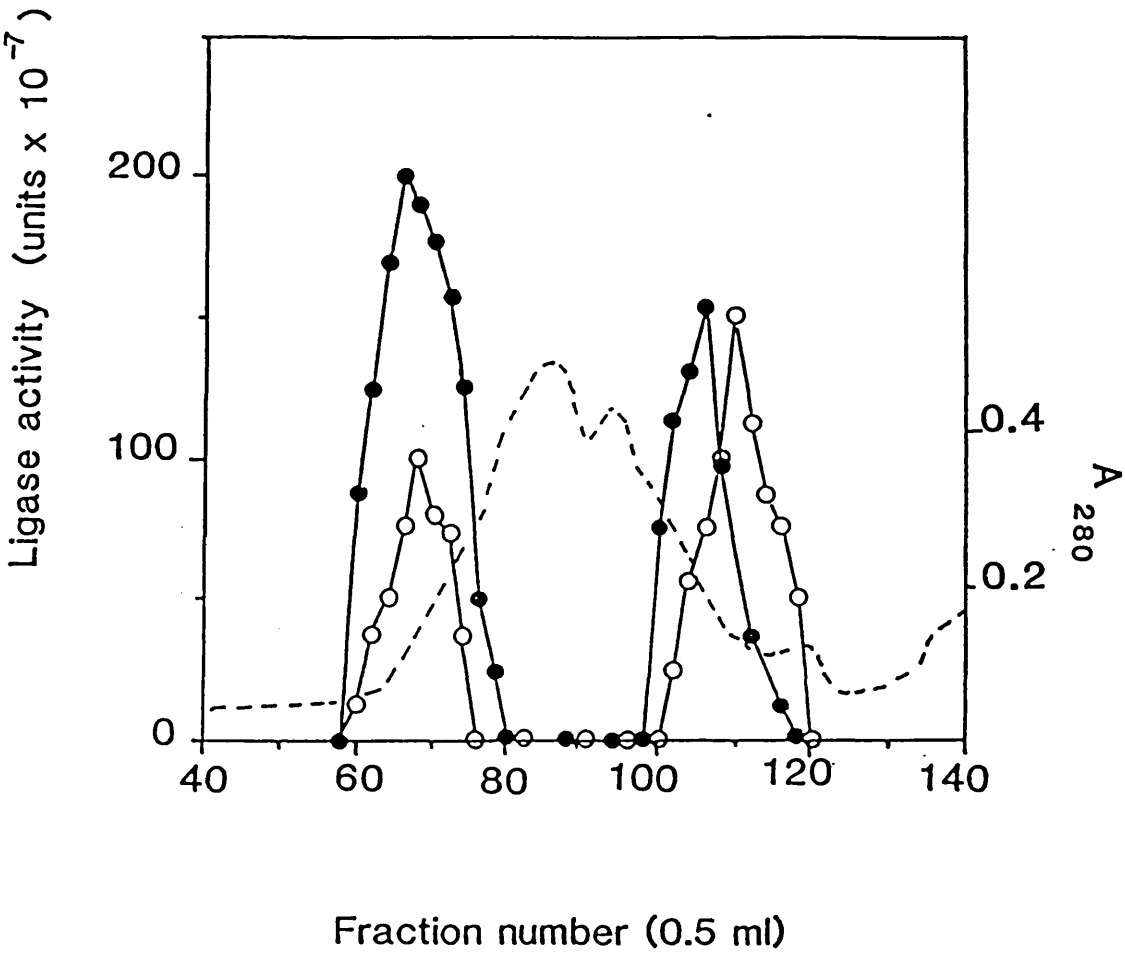


Fig. 21 DNA ligase activities in the Bloom's syndrome cell line GM3403 and Xeroderma pigmentosum group A cell line GM2250

Proteins were eluted from an AcA34 column and assayed for ligase activity using poly(dA)·oligo(dT) substrate. Open circles denot results with GM3403 and closed circles those with GM2250. Dashed line represents A_{280}.

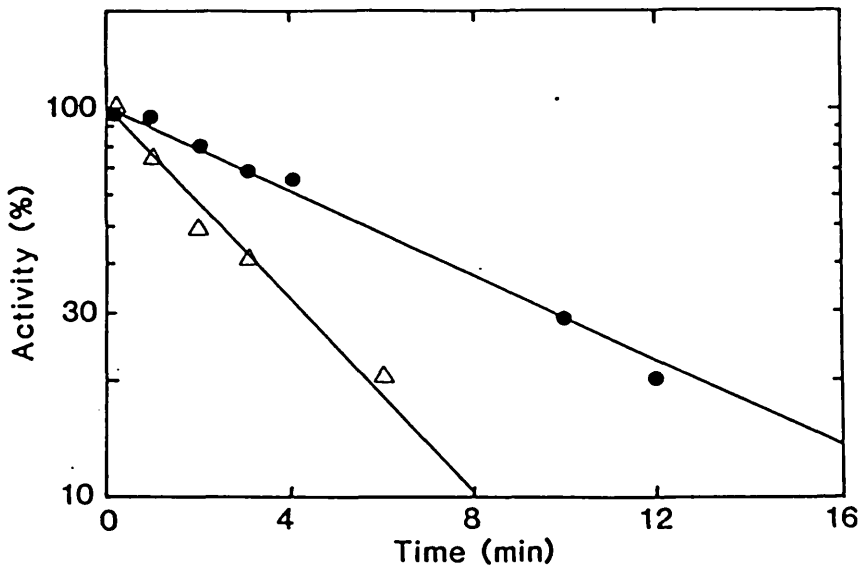


Fig. 4.22 Heat lability of DNA ligase I in Bloom's syndrome

Ligase I that had been purified ~20 fold (Section 2.) from Raji cells (●) and Bloom's syndrome cell line GM3403 (Δ) were heated to 50°C. Aliquots (50μl) removed at predetermined time intervals and assayed for enzyme activity.

both cell types was slightly more labile than in the previous experiments, presumably because the protein concentrations were much lower, 15μg ml^{-1} (Fig. 4.22).

4.1.10 Biochemical Parameters of Bloom's syndrome cell lines

DNA ligase I activities from size fractionated cell extracts of xeroderma pigmentosum group A, 1032 and GM3403, were assayed in the presence of a range of NaCl concentrations. DNA ligase I from all cell extracts was found to be inhibited at NaCl concentrations of 100mM and above (Fig. 4.23), similar to the inhibition observed when DNA ligase I was purified from calf thymus (Soderhall & Lindahl, 1976). Ligase reactions were carried out at pH7.8 and in the presence of 10mM $MgCl_2$ and 1mM ATP for optimal enzyme activity. Therefore, pH dependence of the enzyme and the ability of the enzyme to use Mn^{2+} instead of Mg^{2+} as a cofactor were also examined. However, in each case no difference was detected.

4.1.11 Mex Phenotypes

Human lymphoid cell lines may be of either the Mex^+ or Mex^- phenotype the latter being anomalously sensitive to alkylating agents (Sklar & Strauss, 1981). Mex^+ cells contain between 10,000 - 100,000 molecules of the DNA repair enzyme O^6-methylguanine-DNA methyltransferase, whereas Mex^- cells have no detectable activity (Harris et al., 1983). This enzyme acts by repairing the mutagenic lesion O^6-methylguanine in DNA, by the transfer of the methyl group

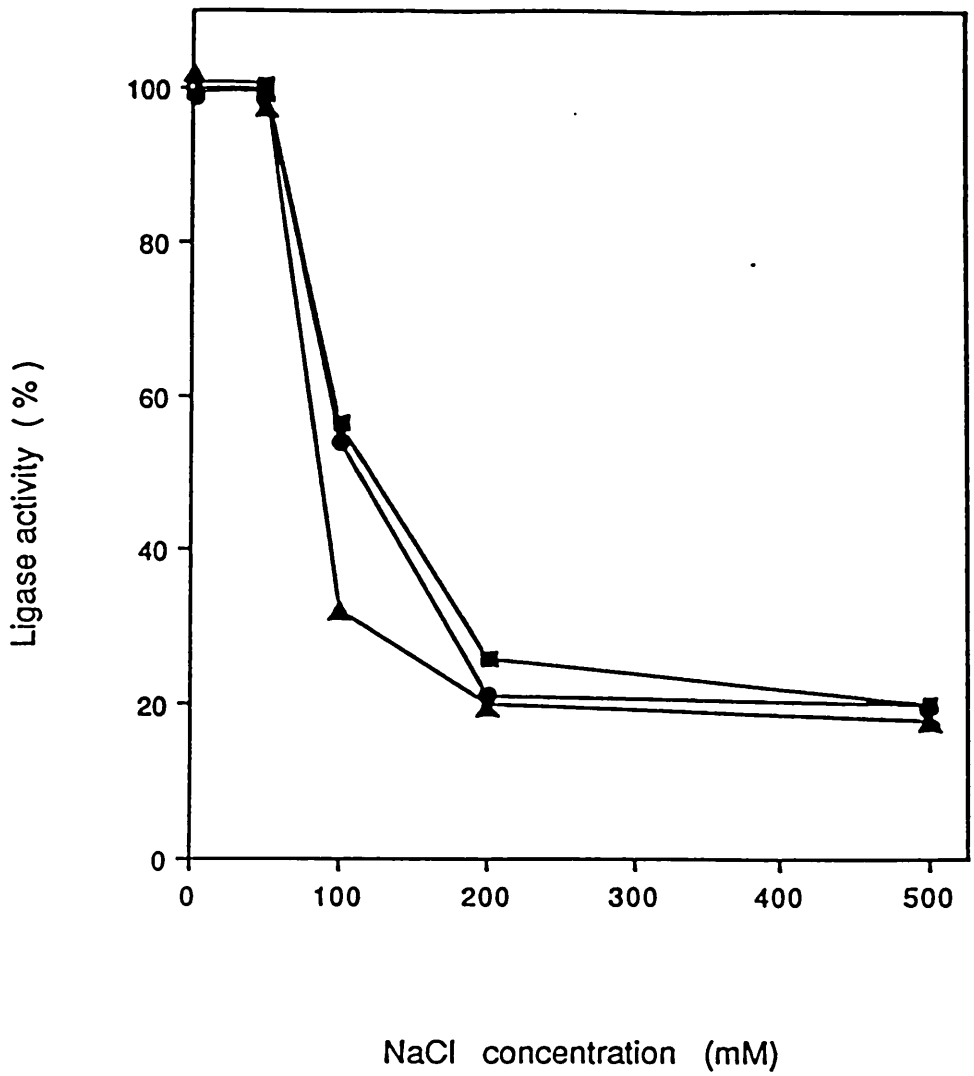


Fig. 4.23 Inhibition of DNA ligase I from Bloom's syndrome by increasing NaCl concentrations

DNA ligase I peak fractions from Bloom's syndrome cell lines W1032 (●), GM3403 (▲) and Raji(■)cells were assayed in the presence of increasing concentrations of NaCl.

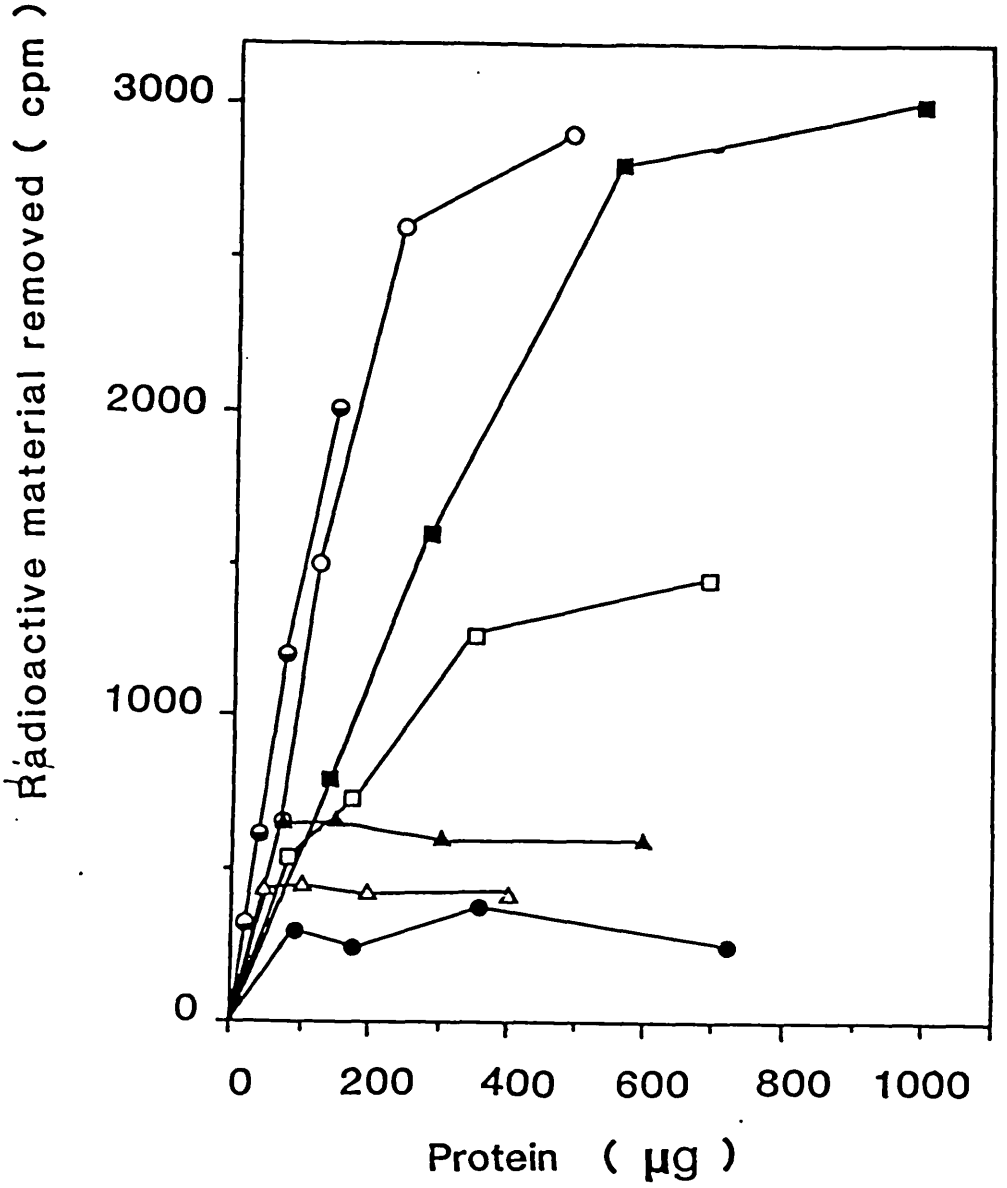


Fig. 4.24 O^6-Methylguanine-DNA methyltransferase activities in Bloom's syndrome cell lines

Cells were extracted and enzyme activities determined as described employing as substrate DNA containing scattered [^{3}H-methyl] O^6-methylguanine residues (Section 2.34). Symbols denote Bloom's syndrome cell lines GM8505 (⊖), GM3403 (●), W674 (Δ), 1032 (▲), D86-1-2 (■), Xeroderma pigmentosum group A (o) and Bloom's syndrome 1004 (□).

to a cystein residue in the protein, which undergoes suicide inactivation in the process. It has been reported that Mex^- cells show delayed joining of strand interruptions (Mattern et al., 1981). Therefore, in order to test whether there were any correlation between this phenotype and Bloom's syndrome, six Bloom's syndrome cell lines were assayed for 0^6-methylguanine-DNA methyltransferase activity.

There was apparently no connection between the Mex phenotype and DNA ligase I activity. The Bloom's syndrome lines GM8505, D86-1-2 and 1004 were Mex^+, W674, GM3403 and 1032 were Mex^- (Fig. 4.24).

4.2 Summary

The data presented here indicates a consistent correlation between a defect in DNA ligase I and Bloom's syndrome. Cell lines independently derived from eight Bloom's syndrome patients have been studied and in each case the DNA ligase I was shown to have altered properties. Two different structural alterations in DNA ligase I were apparent and there appears to be a correlation in these alterations and the ethnic origins of the Bloom's syndrome patients.

The lymphoblastoid cell line GM4408 derived from a patient clinically diagnosed as Bloom's syndrome was the only line to exhibit normal levels of DNA ligase I and a normal frequency of SCE. Restriction fragment length polymorphism and isoenzyme analysis between GM4408 and the fibroblast line

GM3498 (derived from the same patient which has a high SCE frequency) indicated that this cell line is a true revertant. Therefore, it should be possible to locate the position of the mutation by comparing the sequences of DNA ligase I gene from the two cell lines. No other differences were found between DNA ligase I from Bloom's syndrome cells and that from control cells. In addition, the DNA ligase I protein from Bloom's syndrome cells showed similar exclusion upon gel filtration as observed in normal cells.

In conclusion, the data presented in this section support a model in which Bloom's syndrome may be due to a missense mutation in the structural gene for DNA ligase I, which thus results in a malfunctioning version of this essential enzyme.

Chapter 5

5.1 Introduction

In the work described, I have attempted to complement the DNA ligase I defect in Bloom's syndrome by transfection of an expression vector containing the Saccharomyces cerevisiae DNA ligase gene cdc9. Such an approach has been used to stably integrate the E. coli ada$^+$ gene in CHO cells (Kataoka et al., 1986). In these experiments, the CHO cell lines used were Mex$^-$ (i.e. did not contain an active DNA methylguanine 0^6-methyltransferase, Section 4.1.10) and were, therefore, sensitive to both killing and mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Transformation of these CHO cell lines with the E. coli ada$^+$ gene conferred resistance to MNNG, indicating expression of the 0^6-methylguanine-DNA methyltransferase (Kataoka et al., 1986).

The two yeasts S. cerevisiae and Schizosaccharomyces pombe differ substantionally in the organization of their cell cycles (Nurse, 1985). In S. cerevisiae, DNA ligase is periodically expressed during the cell cycle with the level of the cdc9 transcript increasing manyfold in late G1 and reaching a peak at the G1/S phase boundary (White et al., 1986). In contrast, the S. pombe DNA ligase is present at a constant level throughout the cell cycle (White et al., 1986). However, S. pombe DNA ligase mutants (cdc17) can be complemented with the cdc9 gene of S. cerevisiae (Barker & Johnston, 1983; Johnston et al., 1986), although the cdc17 gene fails to complement cdc9 mutation in S. cerevisiae. Unlike S. cerevisiae, S. pombe DNA contains introns, and

thus, the inability to complement the cdc9 mutation with cdc17 may be due to the inability of S. cerevisiae to splice the S. pombe RNA. Although there have been reports of S. pombe correctly translating mammalian cDNA (Lee & Nurse, 1987), it is not known if the reverse is possible.

The cdc9 gene was chosen to attempt complementation of the defect in Bloom's Syndrome. This gene encodes the S.cerevisiae DNA ligase and presumably could be directly transcribed and translated by mammalian cells without splicing.

5.2 Construction of Plasmid pAW1

The plasmid pR12Sclig4, encoding the DNA ligase gene from S. cerevisiae was a gift from Dr. L. Johnston, N.I.M.R., Mill Hill, London, NW7. The plasmid pAW1 was constructed by inserting a 2770bp fragment containing the cdc9 gene directly into the HindIII site of pSV2Neo, bringing it under the control of the SV40 early promoter (fig. 5.1). The plasmid pSV2Neo conveys resistance to the antibiotic geneticin (G418). Mammalian cells are sensitive to this antibiotic and the response time for cell killing appears to correlate with growth rate (Southern & Berge, 1982). Both the Bloom's syndrome fibroblast cell line GM3402C and the control cell line, GM730, were similarly sensitive to this compound and required concentrations of 400μg/ml for approximately 7 days for cell death to occur (Fig. 5.2).

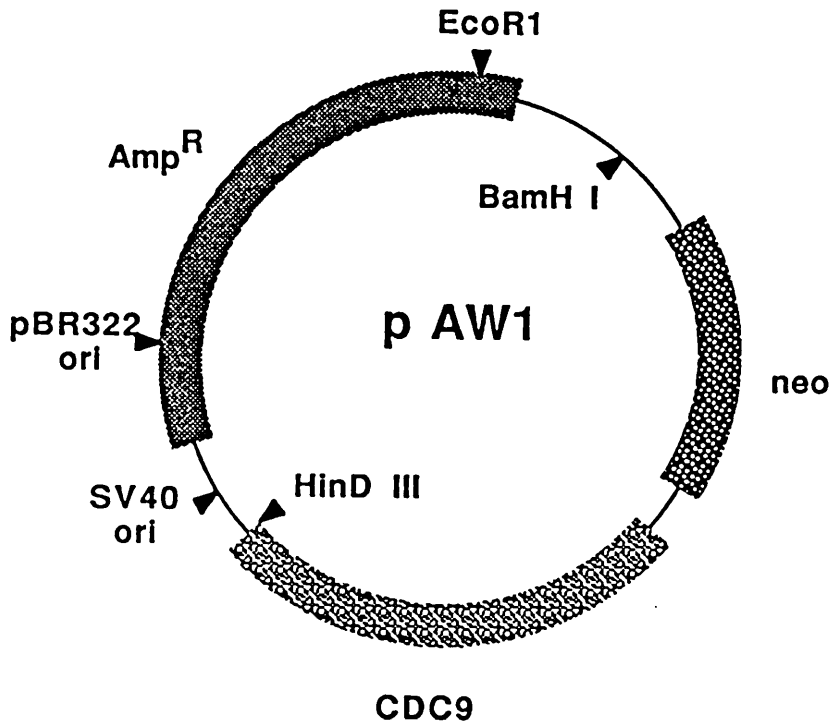


Fig 5.1 Structure of the pAW1 plasmid.

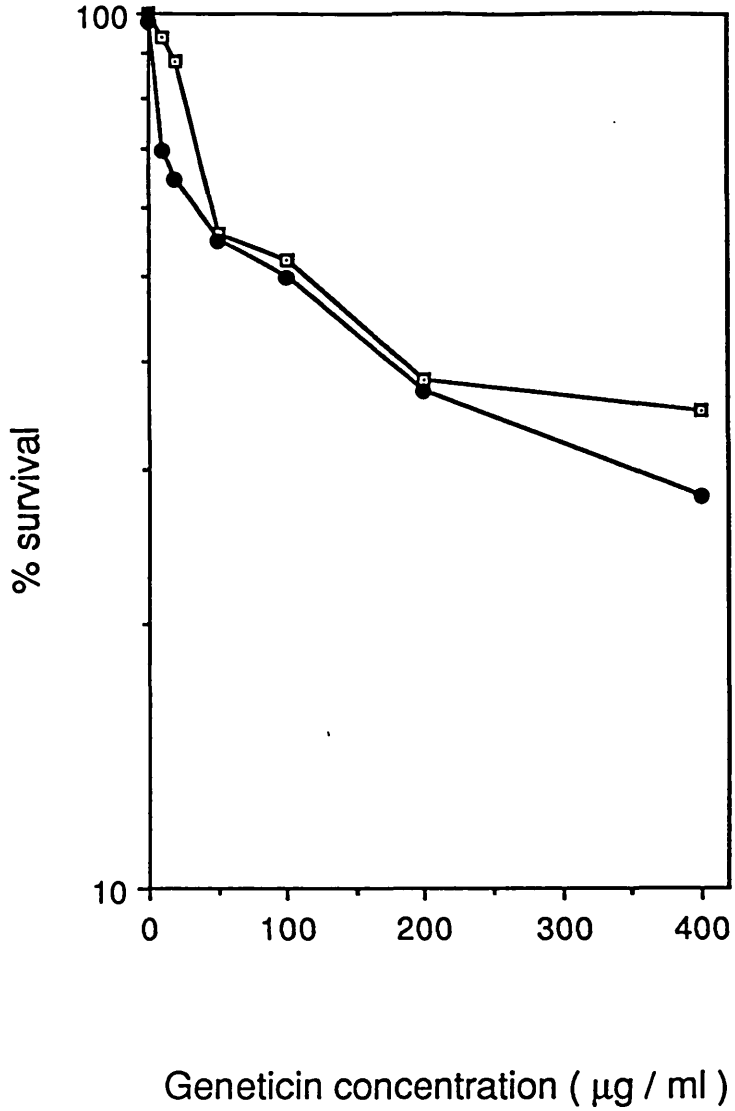


Fig. 5.2 The effect of Geneticin as a function of concentration on Bloom's syndrome, GM3402C (●) and normal GM730 fibroblasts (□). The appropriate number of cells ($1x10^2$-$3x10^4$) were plated in 90mM dishes. After attachment cells were treated with continuous 7 day incubation with the various amounts of geneticin indicated.

5.3 Sensitivity of the Bloom's syndrome fibroblast cell line GM3402C to DNA damaging agents

In order to provide a secondary selection assay for complementation, the Bloom's syndrome cell line GM3402 was tested with a number of DNA damaging agents prior to transfection with pAW1.

Krepinsky et al. (1977) have demonstrated that Bloom's syndrome lymphocytes are sensitive to ethylmethane sulphonate (EMS) and that this compound increases the frequency of SCE by a further six fold. However, the survival difference between the Bloom's syndrome line GM3402 and the control line was small (Fig. 5.3), and GM3402C was only approximately two fold more sensitive. The EMS resistant colonies formed by GM3402 were found to be very diffuse, and scoring for colonies was rather inaccurate. Therefore, for the other cell survival experiments presented here, the ability of cells to incorporate radioactive thymidine [as described in Section 2. (Goss & Parsons, 1977)] was used to measure cellular sensitivity after mutagenic treatment. It has been reported by Ishizaki et al., 1981, that Bloom's syndrome fibroblasts are sensitive to killing by mitomycin C, however, these lines exhibit a wide range of sensitivities to this compound. The cell line GM3402C was more sensitive to mitocycin C than GM730 (Fig. 5.4), however, the difference between these two cell lines was not sufficient to use this chemical as a method for selection of secondary transformants.

The cell line 46BR is very sensitive to

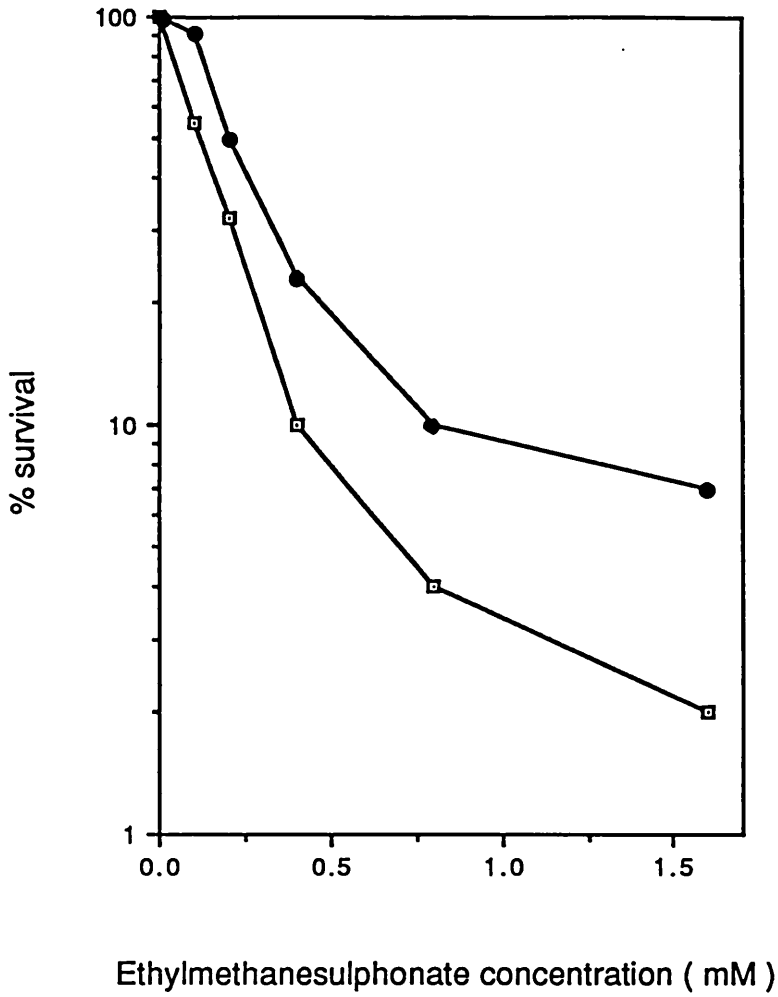


Fig. 5.3 EMS survival curves of normal and Bloom's syndrome fibroblasts

The appropriate number of GM3402 and GM730 cells ($1x10^2$-$3x10^4$) were plated in 90mm dishes. After attachment, cells were treated with the various amounts of EMS indicated. Bloom's syndrome cell line GM3402, ●, normal fibroblast cell line GM730, ⊡.

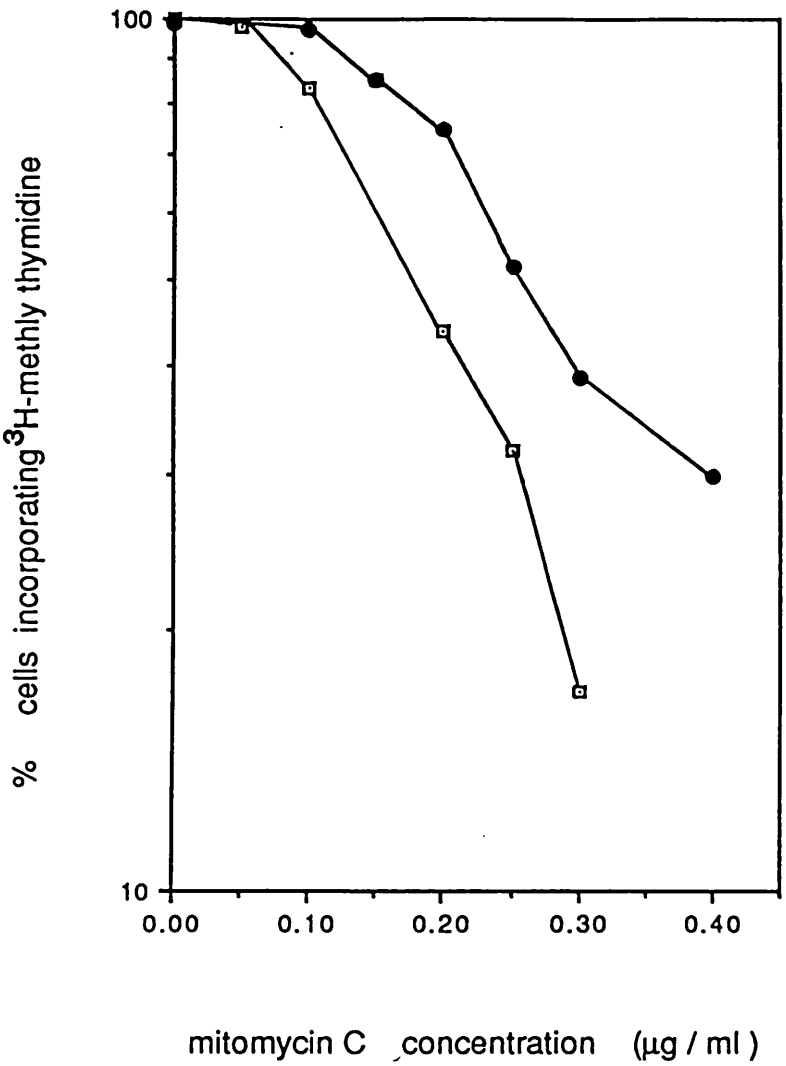


Fig. 5.4 Cell killing in response to mitomycin C
$1x10^4$ cells of either GM730 or GM3402 were plated in 6 well dishes. After attachment, cells were treated with various concentrations of mitomycin C for 1h at 37°C. Bloom's syndrome cell line GM3402 ●, and normal fibroblast line GM730 ▫,

3-aminobenzamide, such that these cells show less than 10% survival at concentrations that have no effect on normal cells (Teo et al., 1983). In view of the clinical similarity between Bloom's syndrome and 46BR and the delayed joining of Okazaki fragments occurring in 46BR, the sensitivity of GM3402C to 3-amino benzamide was tested. Whilst, GM3402C was more sensitive than the control cell line GM370, it was not as sensitive to killing by this compound as 46BR (Fig. 5.5).

Bloom's syndrome fibroblasts show a dose dependent sensitivity to both killing and SCE induction by bromodeoxyuridine (BrdU), (Yamamoto & Fujiwara, 1986). However, although a large proportion of the SCE in Bloom's syndrome cells are apparently caused by BrdU, even at low concentrations of this compound (0.1μg/ml) the SCE frequencies are 30 per cell, a five to ten fold higher level than that observed in normal cells (Shiriashi & Ohtisuki, 1987) although sensitivity to killing by BrdU depends upon the cell line studied (Yamamoto & Fujiwara, 1986). The cell line GM3403C was only slightly more sensitive than the control cell line GM730 to BrdU, such that a two fold difference in cell survival was observed (fig 5.6).

The Bloom's syndrome cell line GM3402 appears to be susceptible to killing by a number of DNA damaging agents, but a sufficiently large difference was not observed between this line and control cells to use the compounds tested to screen for secondary transformants. The effect of ultraviolet on GM3402C should also be examined, as it has been shown by Giannelli et al. (1977) that several Bloom's

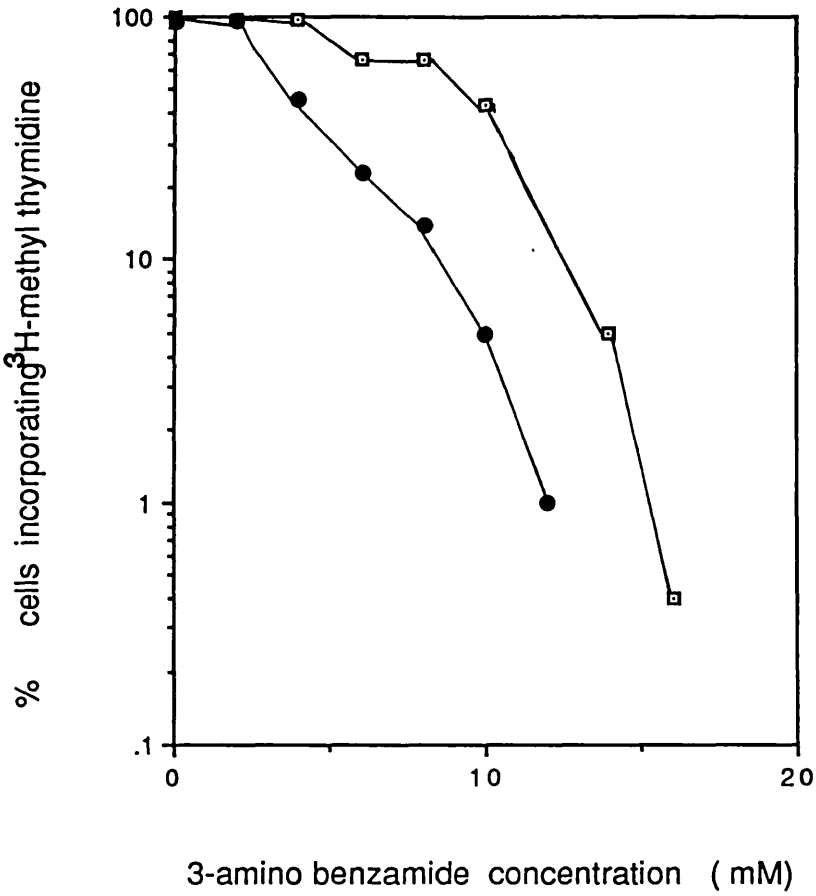


Fig. 5.5 Cell Killing in response to 3-aminobenzamide
Cells ($1x10^4$ of either GM3402 or GM730) were plated into 6 well dishes and treated continuously for eight days with various concentrations of 3-aminobenzamide. Symbols denote Bloom's syndrome cell line GM3402 (●), normal fibroblast line GM730 (⊡).

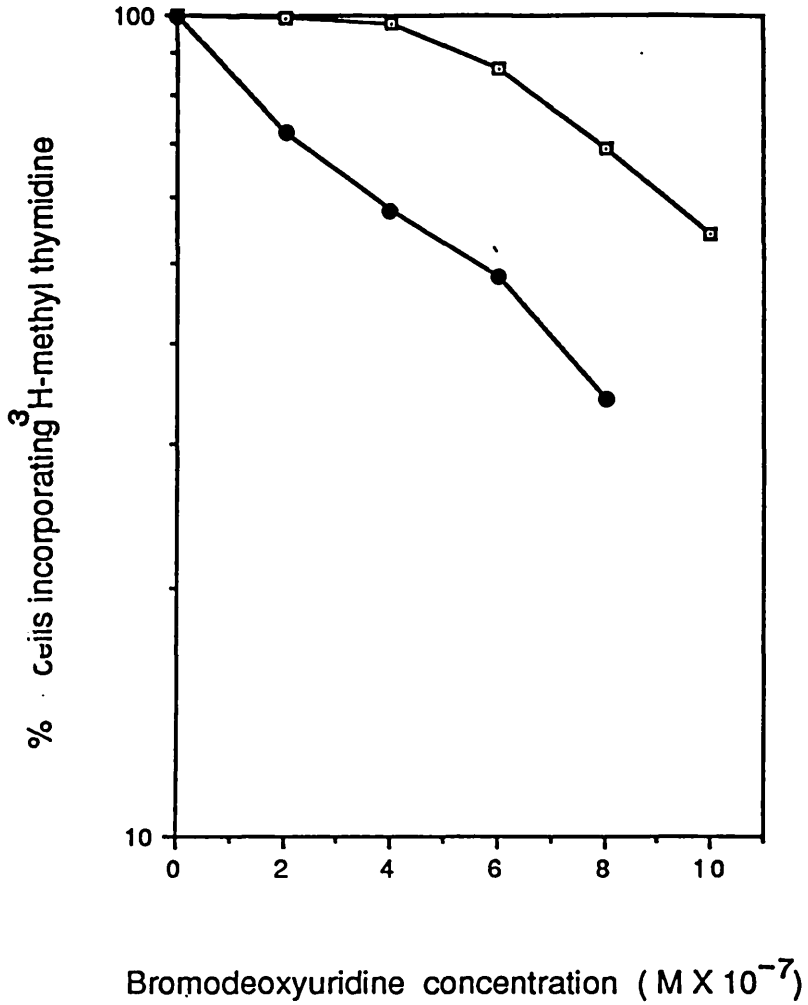


Fig. 5.6 The response of Bloom's syndrome and normal fibroblasts to bromodeoxyuridine

The appropriate number of cells ($1x10^4$ of either GM3402 or GM730) were plated in 6 well dishes. After attachment cells were treated with continuous 8-day incubation with the various amounts of BUrd indicated. Blooms's syndrome line GM4302,●, normal fibroblast line GM730 ⊡.

syndrome cell lines are slightly sensitive to this agent.

Alternatively, the sensitivity of GM3402C to these compounds could be re-examined at a higher temperature, that is, at 39°C. The SCE frequency in Bloom's syndrome cell lines is affected by temperature, such that at higher temperatures the frequency is particularly elevated (West et al., 1981). Therefore, it is possible that the cytotoxic effects on Bloom's syndrome cells of the chemicals tested might be enhanced under these conditions.

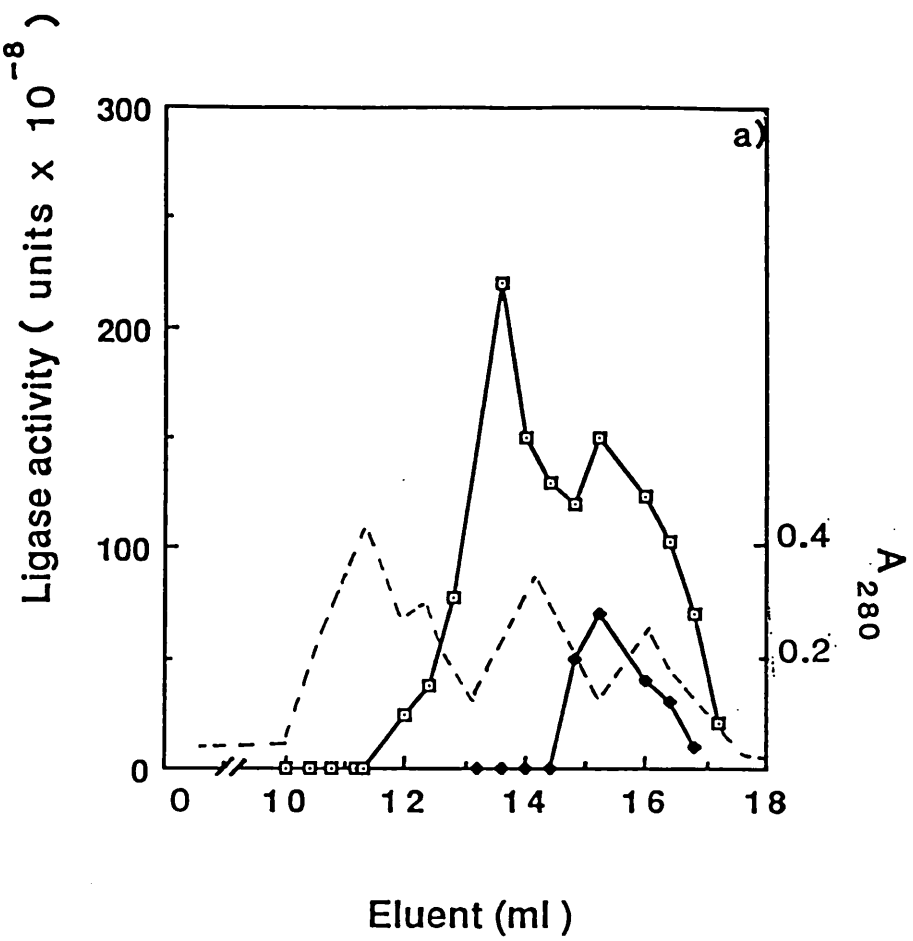
5.4 Transformation of CHO and GM3402 cell lines with the dual expression vector pAW1

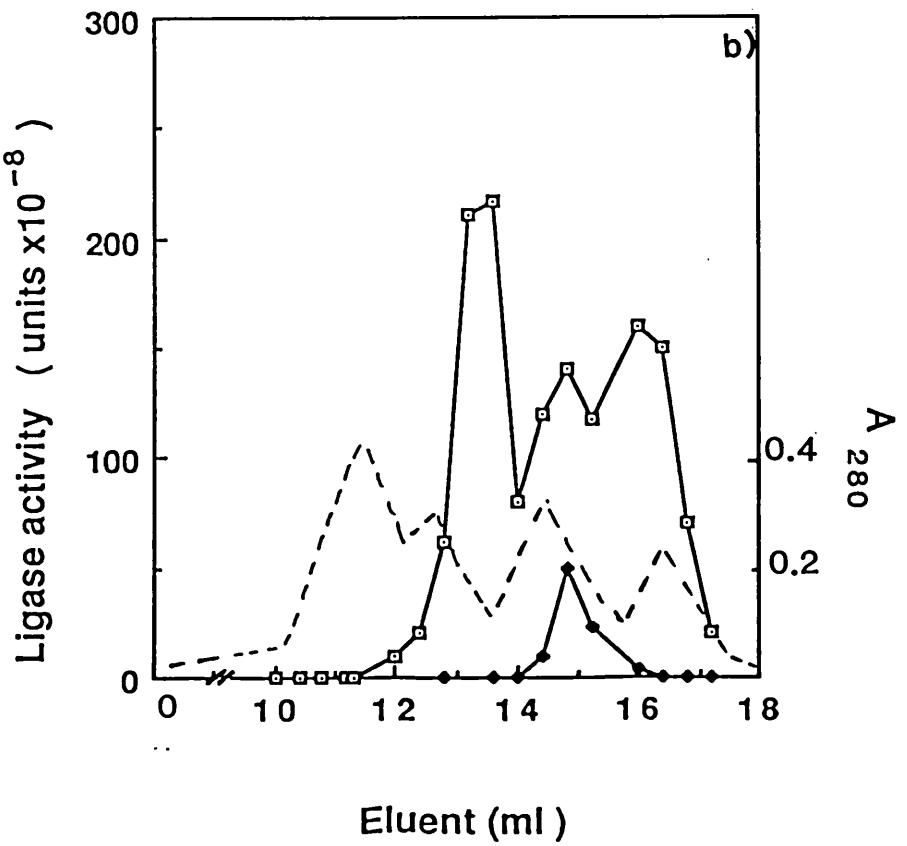
Following the transfection of pAW1 into CHO cells, cloned cell lines were obtained which were resistant to the antibiotic geneticin (G418), and therefore expressed the Neo gene. The cells were transfected with plasmid DNA by using Polybrene (see Section 2.75), and after two weeks yielded colonies at a frequency of 0.014% for 20μg of pAW1 and 0.06% for 20μg of pSV2Neo. The cloned cell lines were expanded to obtain a sufficient amount of material to determine DNA ligase activity. Cells were extracted and the DNA ligase activities separated as previously described (Section 2.61). A DNA ligase peak of ~60kDa was observed in the cloned CHO cell line which was not present in the control line (Fig. 5.7). This could be due to a truncated active form of the S.cerevisiae DNA ligase, which would indicate that the cdc9 gene was functionally expressed.

No geneticin resistant colonies were formed by cells

Fig. 5.7a DNA ligase activities in CHO cells
Cells were extracted and enzyme activities separated on a FPLC Superose 12 column as described previously. DNA ligase I and II were assayed using poly(dA)·oligo(dT) substrate (□), and the position of ligase II was verified using the hybrid substrate poly(rA)·oligo(dT)(♦) Dashed line represents A_{280}.

Fig. 5.7b DNA ligase activities in CHO cell line transfected with pAW1
Legend and symbols as in Fig. 5.7a.


a)
Ligase activity (units x 10^{-8})
300
200
100
0
0.4
0.2
A_{280}
0
10
12
14
16
18
Eluent (ml)


b)
Ligase activity (units x10^{-8})
300
200
100
0
0.4
0.2
A_{280}
0
10
12
14
16
18
Eluent (ml)

from the Bloom's syndrome cell line GM3402C. Although three different transfection techniques were tried (Section 2.75), it is possible that the plasmid DNA did not enter the cells. Another explanation for these negative experiments could be that the plasmid DNA was not stably integrated into the host chromosome. As DNA ligase must, presumably, be required for stable integration of DNA, the DNA ligase I defect in Bloom's syndrome cells could render this process uneffective.

5.5 Summary

No transformants were obtained with the Bloom's syndrome line, GM3402, in transfection attempts with the plasmid pAW1 carrying the yeast DNA ligase gene. It would obviously be important to test the efficiency of the transformation procedure, so that conditions can be optimized. However, it is possible that the pSV2Neo entered the cells, but was not stably integrated, or alternatively, perhaps Bloom's syndrome cells cannot be selected with geneticin. In both these cases, it would be necessary to subclone the cdc9 gene into a different vector such as pSV2CAT or pSV2GPT that express as an enzyme readily assayed.

Since DNA ligases have an essential function in DNA replication perhaps acting in concert with other enzymes, it is possible that the Bloom's syndrome defect cannot be complemented by the cdc9 gene. Once the mammalian DNA ligase gene has been cloned, it would be of interest to transfect this gene into Bloom's syndrome cells.

Chapter 6

Discussion

6.0 Discussion

This thesis describes the development of specific assays for each of the two mammalian DNA ligases, which has allowed their distinction and separate analysis. These assays have enabled DNA ligase activities to be determined in cell lines derived from inheritable cancer prone syndromes which led to the discovery of a DNA ligase I deficiency in Bloom's syndrome.

6.1 Evidence for the existence of two mammalian DNA ligases

It has been shown here that the two mammalian DNA ligases have different substrate specificities, indicating that they are separate entities. Hence, DNA ligase I is a 200kDa protein which is capable of joining strand breaks in 'nicked' plasmid DNA, blunt ended DNA, and double-stranded polynucleotides consisting of poly(dA)·^{32}P(oligo dT)$_{25-30}$. DNA ligase II has a molecular weight of ~80kDa and seals strand breaks in 'nicked' plasmid DNA, the polymer substrate poly(dA)·^{32}P(oligo dT)$_{25-30}$ and also the hybrid polymer substrate of poly(rA)·^{32}P(oligo dT)$_{25-30}$. However, neither of these enzymes act as RNA ligases, and they cannot join strand breaks in poly(I)·poly(C) substrates (Soderhall & Lindahl, 1976).

Due to the frequently observed size heterogeneity of partly purified preparations of DNA ligase I, a number of

reports have disputed the existence of two DNA ligases. Teraoka & Tsukada (1982) initially reported their inability to find DNA ligase II in calf thymus cell extracts as evidence for there being no such enzyme. In addition, Mezzina et al., (1985), after partial separation of the two activities, found a number of common bands on a DNA ligase 'activity gel', and proposed that DNA ligase II was derived from DNA ligase I. A possible explanation of these results could be that their DNA ligase II preparations were contaminated with DNA ligase I, and the conditions used to renature the enzymes after polyacrylamide gel electrophoresis may not have been adequate to regenerate ligase II activity. Therefore, their "ligase II" activity could have been due to active fragments of DNA ligase I. Teraoka & Tsukada (1985) showed that DNA ligase I is susceptible to proteolytic cleavage, as a monospecific antibody directed against this protein precipitated a 200kDa peptide in crude cell extracts, which was degraded to a 130kDa peptide in vitro as shown by pulse chase labelling experiments.

Soderhall & Lindahl, 1975, obtained a ligase I antibody which did not inhibit DNA ligase II. Furthermore, Teraoka et al. (1986) purified DNA ligase II ~4000 fold as a 68kDa protein, and antibody directed against this protein cross reacted with DNA ligase II, but not DNA ligase I. It may be concluded that DNA ligase I and II show no immunological cross-reaction. Hence, the

different substrate specificities of I and II coupled with the immunological data (Soderhall & Lindahl, 1973; Teraoka & Tsukada, 1985; Teraoka et al., 1986) show unequivocally that they are separate entities.

Although certain lines of evidence have indicated that DNA ligase I is involved in DNA replication (Soderhall, 1976; Chan & Becker, 1984) there is no known function of DNA ligase II. The activity of DNA ligase II does not increase on cellular proliferation and it has been suggested that this enzyme is not involved in DNA replication (Soderhall, 1976, Chan & Becker, 1984). The different substrate specificities of this enzyme do not give any indication as to its physiological role, except that as it will ligate hybrid DNA·RNA substrates, it has a less stringent substrate specificity in this regard than DNA ligase I. It has been proposed that DNA ligase II may have a function in DNA repair (Creissen & Shall, 1982), although there has been little evidence to support this theory.

If DNA ligase II were involved in DNA repair, it is possible that an inheritable human syndrome might have a reduced level of this enzyme. Such a syndrome would be expected to be sensitive to a wide range of DNA damaging agents, particularly those which cause DNA strand breaks. In the inheritable cancer prone syndromes screened here, no evidence was obtained to indicate a defect in DNA ligase II. There have been preliminary reports on

decreased levels of DNA ligase activity in cell lines derived from Fanconi's anaemia and Cockayne's syndrome, although the differences observed between the DNA ligase activities in those experiments may reflect the slow growth of these cell lines (Hirsh-Kauffmann et al, 1978; Schweiger et al., 1986) compared to normal cells. Furthermore, in these experiments the two DNA ligases were not separated prior to assay and it is unclear which activity might have been deficient. In the experiments presented in this thesis, one example of a cell line derived from both a Fanconi's anaemia and a Cockayne's syndrome patient was examined for deficiencies in DNA ligase I and II. Although no differences were observed when compared to normal cells, this may not reflect the general situation since only a limited number of lines were available. In addition, in cell lines derived from ataxia telangiectasia and Fanconi's anaemia, Coquerell & Weibeizhn (1981) have reported impaired rejoining of DNA strand breaks caused by γ irradiation. These results do not necessarily indicate that these cells are deficient in a DNA ligase, but possibly in some other enzymic step of excision repair. The types of DNA damage caused by γ irradiation has been investigated by Henner et al. (1982), who found that three types of DNA strand breaks occur. At the 3' and 5' terminus of radiation induced fragments, phosphoryl groups were observed. However, also at the 3' terminus the authors found a group which was neither a

phosphoryl group nor a hydroxyl group, but had properties consistent with a portion of the deoxyribose esterified to a 3′-terminal phosphate. It was suggested that this probably was a glycolic acid group which was esterified to the 3′ phosphate. All the assays for DNA ligase activity measure the ability of the enzyme to join 3′-5′ phosphodiester bonds. It is, therefore, not known if a DNA ligase could rejoin a glycolic acid esterified at 3′ phosphate residues. A deficiency in this type of ligase activity would not be detected by standard DNA ligase assays.

6.2 A DNA ligase defect in Bloom's syndrome

6.2.1 Two structural alterations of DNA ligase I in Bloom's syndrome

The evidence presented in Chapter 4 shows that cell lines derived from Bloom's syndrome patients have a defect in DNA ligase I. Two types of defects have been found, which presumably represent two different mutations in the structural gene for DNA ligase I.

In the five cell lines derived from patients of Ashkenazi Jewish origin, the DNA ligase I activity is reduced by 70% and the residual remaining enzyme is twice as heat labile as that in the control cells. This is termed a type 1-1 defect. Such a defect is consistent

with the hypothesis that most Jewish individuals with Bloom's syndrome are descendants of a single ancestor who lived in Poland (German, 1984). A similar deficiency is observed in the cell line 1004 derived from a Mennonite patient. The two cell lines AA87-5-1 and D86-1-2 with a low frequency of SCE were also found to have reduced DNA ligase I activities. The reduced frequency of SCE in these two cases is not, therefore, due to a back mutation, but could be caused by the compensatory overproduction of another replicative function.

Three cell lines were derived from non-Ashkenazi Jewish patients; 1032, GM8259A and 46BR (see section 1.53), the ethnic origin of which were French Canadian, Japanese and Anglo-Saxon, respectively. In these cell lines a different type of defect was present, which is manifest in an enzyme that dimerizes, to give a large proportion of the active DNA ligase I with a molecular weight of ≃400kDa. These dimers are present at physiological salt concentrations but can be dissociated at high ionic strength. The DNA ligase I from these cell lines is not heat labile. This type of alteration was termed type 1-2. The two types of structural defects observed here (1-1 and 1-2), may result from the expression of two different point mutations.

Chan et al. (1987) also report a structural alteration in DNA ligase I activity in Bloom's syndrome. The authors observed three peaks of DNA ligase activity of

molecular weights 480, 240 and 80kDa in their control cell lines. These would correspond to dimers and monomers of ligase I and DNA ligase II, respectively. In Bloom's syndrome lines, they claim to find two differences; (i) a shift of the 480kDa peak to 400kDa and (ii) a 50% reduction in the amount of DNA ligase I in the dimer, but no reduction in the ligase I monomer. It is difficult to reconcile these results with the data presented here. The main differences between these experiments is that Chan et al. (1987) prepare extracts in a buffer of high ionic strength and do not remove the DNA before size fractionating their extracts, thus it is possible that the 'dimers' observed result from DNA ligase bound to DNA. The 'dimers' were found in the void volume of the column, so it is unclear how it was possible to estimate their molecular weights.

6.2.2 DNA Ligase Mutants

DNA ligase mutants of bacteria and yeast have been extensively studied, and exhibit phenotypic characteristics similar to Bloom's syndrome cell lines. In this respect, DNA ligase mutants from E.coli, S.cerevisiae, and S.pombe have been isolated which have heat labile DNA ligases, show a delayed rate of Okazaki fragment joining, have increased sensitivity to killing by ultraviolet light and alkylating agents and exhibit an elevated rate of recombination (Table 6.1).

Table 6.1

DNA Ligase Mutant	DNA Ligase Defect	Delayed rate of DNA synthesis	Increased frequency of recombination	Cellular Sensitivity UV irradiation	EMS or MMS
E.coli lig4	Heat labile enzyme 35% activity at 30°C to 1% at 42°C (1, 2).	At 42°C seals Okazaki fragments 10-fold more slowly (2).	cells display hyper-rec phenotype (3).	slight sensitivity, at 42°C. Cells loose their low dose plateau (2).	slight sensitivity to MMS (2).
E.coli Ligts7	Heat labile enzyme 25% activity 15°C to < 1% at 42°C (2,4)	40-fold decrease in the rate of joining Okazaki fragments (4,2).	cells display a hyper-rec phenotype (3).	killed rapidly at at 35.5°C (5).	extreme sensitivity to MMS (6).
E.coli Lig321	No enzyme present at 42°C.	At 30°C a 30-fold decrease in the rate of Okazaki fragment joining (7).	cells display a hyper-rec phenotype (3).	N/D	N/D
S.Pombe CDC17	Heat labile t of 1.4min at 36°C, except ligase mutant M75 (8,9)	Defective in Okazaki fragment joining at the restrictive temperature. Cells are blocked in mitosis (8).	N/D	Sensitive to UV irradiation (8).	N/D
S.Cerevisiae CDC9	Heat labile enzyme (10).	Defective in Okazaki fragment joining (10).	Cells show enhanced mitotic re-combination (11,12).	CDC9-1 is sensitive to killing by UV-irradiation (10) CDC9-9 to γ-irradiation sensitive (13).	CDC91 is sensitive to killing by MMS (14).
Bloom's syndrome	DNA ligase defect. Mutation type 1 causes a heat labile enzyme with at t of 3mins at 50°C.	Delayed rate of DNA synthesis Type 1 cells show delayed rate of of formation of weight DNA (15) and retarded DNA chain growth (16,17) 46BR (type II) shows a delayed rate of Okazaki fragment joining (18).	Cells from BS patients are typified by a high frequency of SCE (19,20).	slight sensitivity to UV-irradiation 215 and 254nm (15,21).	Slight sensitivity to EMS (22).

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10. Johnston & Nasmyth, 1978.
11. Game et al.,
12. Fabre & Roman, 1979.
13. Moore, 1982.
14. Johnston, 1979.
15. Giannelli et al., 1977.
16. Hand & German, 1977.
17. Kapp, 1987.
18. Henson,
19. Chaganti et al., 1974.
20. German et al., 1977.
21. Zbinder & Gerutti, 1980.
22. Krepinsky et al., 1979.

Different E.coli DNA ligase mutants do not complement each other indicating that mutations occur in a single gene. However, there is considerable variation in the severity of the phenotypic defects amongst the E.coli ligase mutants. For example, at the restrictive temperature there is no growth of the strain lig321, whereas ligtS7 and lig4 grow with 1% and <1% respectively of DNA ligase activity. Similarly, no complementation has been observed between independently derived cell lines from Bloom's syndrome patients, and fusion of the type 1-1 defect cells with type 1-2 does not reduce the elevated SCE frequency (Weksberg et al., 1987). Although several types have demonstrated that Bloom's syndrome lines exhibit a wide range of sensitivities when exposed to a variety of DNA damaging agents, the cellular levels of DNA ligase I in the different type 1-1 defect lines studied here were similar. As a tight mutation in the gene for DNA ligase I in mammalian cells would presumably be conditionally lethal, the mutation in this gene in Bloom's syndrome cells would be expected to be leaky.

6.2.3 Relationship between the DNA ligase I defect and an elevated SCE frequency in Bloom's syndrome

Certain lines of evidence indicate that the elevated SCE frequency in Bloom's syndrome cell lines may be directly associated with a DNA ligase I deficiency.

Several reports have shown that constraints on cell

growth reduce the SCE frequency in Bloom's syndrome cells. For example, co-cultivation of CHO cells and normal fibroblasts with Bloom's syndrome cells (Van Bull et al., 1987; Rudiger et al., 1980) and a reduction in growth temperature (West et al., 1981) both cause a decrease in the SCE frequency.

It has been suggested that the reduction of the SCE frequency by co-cultivation was due to the transfer of correcting factors (Van Bull et al., 1978; Rudiger et al., 1980). Furthermore, two reports indicate an increase in SCE in normal cells when co-cultivated with normal cells although these differences are too small to be significant (Tice et al., 1978; Emerit & Cerutti, 1980). However, Bryant et al. (1979) showed that in cell hybrids, formed by the fusion of Bloom's syndrome fibroblasts with normal fibroblasts, there was a normal SCE frequency whilst in fused Bloom's syndrome cells alone the elevated SCE frequency was maintained. These authors concluded that the high frequency of SCE in cell lines derived from Bloom's syndrome reflects an intrinsic genetic defect in this disease.

Furthermore, West et al. (1981) postulated that the reduction in SCE frequency in co-cultivated Bloom's syndrome cells could be due to inhibition of their growth by the more actively growing control cells. The authors demonstrated that a reduction in the growth temperature of Bloom's syndrome fibroblasts also decreased the number of

SCE and suggested that both these observations may be caused by a decreased DNA synthesis rate. A defect in DNA ligase I would correlate with these results as slowing down growth may allow sufficient time for the enzyme to correctly join Okazaki fragments without delay. Alternatively, a decrease in the growth temperature may increase the stability of DNA ligase I in the Bloom's syndrome cells, since it has been demonstrated here that in this syndrome the enzyme is twice as heat labile as in control cells.

It has been suggested that the elevated SCE frequency in Bloom's syndrome cells might be due entirely to their sensitivity to bromodeoxyuridine (Yamamoto & Fujiwara, 1986; Shiraishi & Ohtsuki, 1987). However, even when a ten times lower concentration of bromodeoxyuridine was utilized to detect SCE, the frequency was 30-60 per cell in Bloom's syndrome fibroblasts, a 6-fold increase over that found in normal fibroblasts. The cell line EM9, an experimentally induced mutant of CHO cells, shows extreme sensitivity to bromodeoxyuridine and also has an elevated frequency of SCE (Thompson et al., 1982). However, fused cell hybrids of Bloom's syndrome and EM9 cells are no longer sensitive to bromodeoxyuridine and have normal SCE frequencies and are, therefore, capable of complementation. This indicates that Bloom's syndrome cells are not deficient in the repair of DNA damage caused by this nucleoside analogue (Ray & German, 1984; Ray et

al., 1987), and the normal DNA ligase activities detected in EM9 cells (Chan et al., 1984) concurrently correct the defective enzyme in Bloom's syndrome.

It is possible to correlate the cellular characteristics observed in Bloom's syndrome and explain many of the differences found in cell lines derived from patients with this disease compared to normal cells by a defect in this one essential enzyme. It has been reported that although Bloom's syndrome cells contain normal levels of the enzymes 0^6-methylguanine-DNA methyltransferase, hypoxanthine-DNA glycosylase and uracil-DNA glycosylase, the regulation of these enzymes in the cell cycle is abnormal (Leim et al., 1986; Dehaza & Sirover, 1986; Gupta & Sirover, 1984; Yamamoto & Fujiwara, 1986). The alterations presented may be explained by the slow growth rate of Bloom's syndrome fibroblasts which is potentially related to the defect in DNA ligase I.

6.3 A DNA ligase I deficiency may contribute to cancer proneness

In Bloom's syndrome, which is typified by a high frequency of SCE and other chromosomal aberrations there is an increase in the incidence of all common cancers (German, 1983). From the work presented in this thesis, we may hypothesize that the DNA ligase I deficiency in Bloom's syndrome contributes towards the high frequency of both SCE and chromosomal aberrations, perhaps as a result

of strand breaks remaining unsealed for extended periods. High levels of homologous recombination could potentially lead to homozygosity of certain oncogenes or contribute towards the mechanisms by which these genes are rearranged and amplified (Kuhn, 1985).

In addition, unsealed strand breaks may explain the increased spontaneous mutation rate in Bloom's syndrome cells (Warren et al., 1981), since extending the lifetime of single stranded groups may lead to an increase in premutagenic base damage (Kondo, 1973). A further interesting observation arises from a study of a negroid Bloom's syndrome patient who developed acute lymphocytic leukaemia. Adjacent areas of decreased and increased pigmentation were found on the skin similar to the "twin spots" seen in Drosophila (Festa et al., 1979). In Drosophila, such spots indicate increased somatic cell recombination. It was suggested that in Bloom's syndrome the areas of altered pigmentation were analogous to "twin spots" and that the cells in those areas have become homozygous for a particular gene (Festa et al., 1979). A simple model of how this might occur is shown in Fig. 6.1. A spontaneous point mutation, deletion, or translocation in a somatic cell may produce pre-cancerous cells. This, coupled with a decreased DNA ligase I activity may lead to a high frequency of homozygous chromosome regions via an increased rate of somatic recombination.

The major conclusion from the work presented in this

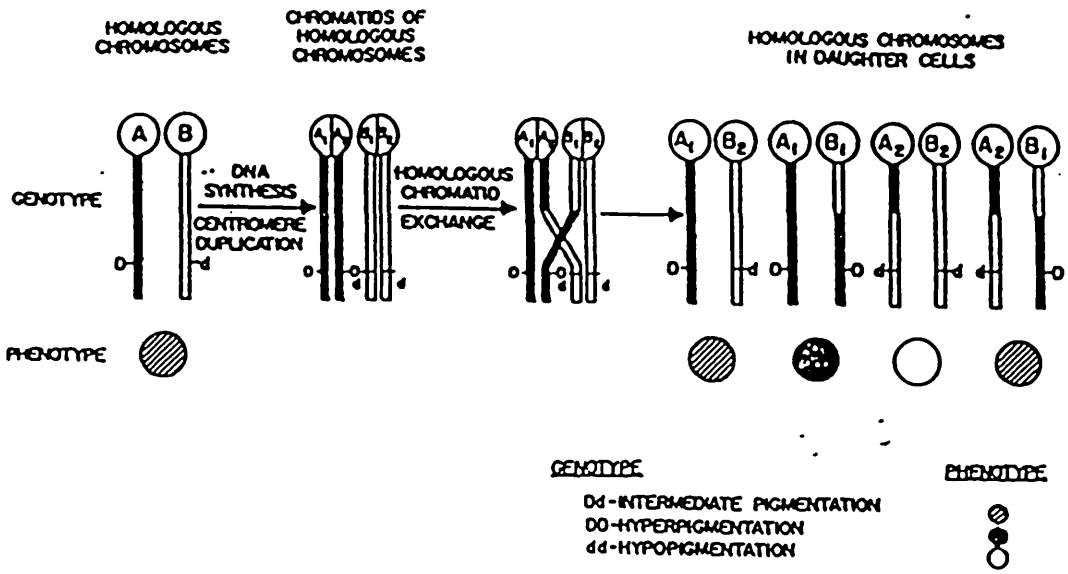

Fig. 6.1 Proposed model for somatic recombination in dermal cells leading to formation of "twin spots"

This figure represents a chromosome fragment from a black individual heterozygous for one of several genes determining intensity of pigmentation. Cells heterozygous (Dd) for that gene are intermediately pigmented when such cells divide somatic recombination during mitosis results in production of cells which are homozygous for each of the alleles producing hyperpigmented (DD) and hypopigmented (dd). In Bloom's syndrome with increased SCE frequency, there would be an increased rate of somatic cell recombination (from Festa et al., 1979).

thesis is that a defect in DNA ligase I occurs consistently in Bloom's syndrome. This may be related to the increased rates of mutagenesis, chromosome breakage and somatic recombination characteristic of the disorder and therefore contribute towards a general predisposition to cancer in Bloom's syndrome patients.

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Different Substrate Specificities of the Two DNA Ligases of Mammalian Cells*

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Mammalian cells contain the DNA ligases I and II. These enzymes show different molecular weights and heat labilities, and antibodies against ligase I do not inhibit ligase II. Here, the nonidentical substrate specificities of the enzymes are described. Under standard reaction conditions DNA ligase I, but not ligase II, catalyzes blunt-end joining of DNA, while ligase II is the only activity that joins oligo(dT) molecules hydrogen-bonded to poly(rA). These differences facilitate the distinction between the two enzymes and should permit further analysis of their functions.

DNA ligases are required for replication and repair processes. Such enzymes have been found in extracts of mammalian cells (for reviews see Refs. 1 and 2). However, the mammalian DNA ligases have not been fully characterized, apparently because of the ready availability and ease of use of microbial enzymes for *in vitro* ligation during molecular cloning. We have previously described two distinct DNA ligase activities in the nuclei of mammalian cells (3, 4), as opposed to the single species found in bacteria and yeast. The two mammalian DNA ligases both require ATP as a cofactor, but they do not cross-react serologically, and only one of them is induced on cell proliferation (4, 5).

The major ligase activity in proliferating mammalian cells is due to a high molecular weight protein termed DNA ligase I. This enzyme is induced up to 15-fold together with DNA polymerase α during rat liver regeneration (5, 6), suggesting a role for ligase I in DNA replication. The enzyme exhibits a short half-life (about 30 min) *in vivo* (7). Ligase I resembles the well-known phage T4 DNA ligase with regard to its biochemical mechanism of action, and covalent enzyme-AMP and DNA-AMP reaction intermediates have been shown to occur (1). The native mammalian enzyme has a molecular weight of 180,000–200,000, as determined by sedimentation velocity and gel filtration measurements (3, 6, 8–10), and the same size is observed for the protein by sodium dodecyl sulfate-gel electrophoresis (7). Size heterogeneity has been reported for ligase I, and the enzyme appears to be present both in monomeric form and as a 400-kDa dimer in cell extracts (6, 11). In addition, ligase I is susceptible to proteolysis and is easily degraded during extraction and purification to large active fragments of molecular weight 90,000–140,000. The activities of such truncated forms are completely inhibited by rabbit antibodies against ligase I (4, 7).

The mammalian DNA ligase II is a smaller protein of molecular weight ~80,000. The enzyme is very heat-labile, exhibits a sharp pH optimum at 7.8, and is not affected by antibodies against ligase I (3, 4). Unlike ligase I, ligase II is not induced on cell proliferation (4–6). Furthermore, on subcellular fractionation DNA ligase II appears to be more firmly associated with the chromatin than does ligase I (4, 6).

EXPERIMENTAL PROCEDURES

Enzyme Purification—DNA ligases I and II were purified from calf thymus as described previously (3, 4) with minor modifications. Briefly, 600 g of fresh thymus glands were disrupted at 0 °C in a Waring blender with 3 liters of a buffer containing 0.1 M NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 μg ml^{-1} each of the protease inhibitors pepstatin, leupeptin, and chymostatin. After centrifugation, nucleic acids were removed by precipitation with 0.5% Polymin P in the presence of 0.2 M NaCl. The activities were purified by ammonium sulfate fractionation (42–67% saturation), dialyzed, applied to phosphocellulose (two parallel columns of 4 × 30 cm each) in 50 mM NaCl, 50 mM Tris-HCl (pH 7.2), 10 mM 2-mercaptoethanol, 1 mM K_2HPO_4, 1 mM EDTA, and eluted step-wise with the same buffer containing 0.5 M NaCl and no EDTA. Both ligase I and ligase II were present in this fraction, which was applied directly to a hydroxyapatite (HA-Ultrogel, LKB Products) column (2.5 × 18 cm) and eluted with a linear gradient (800 ml) of 1 to 200 mM K_2HPO_4 in 0.5 M NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol. This procedure separates ligases I and II (see below, Fig. 2). The two peaks of activity were further purified separately as described previously (4), ligase I by gradient chromatography on phosphocellulose and gel filtration on Sephadex G-150, and ligase II by gel filtration only. Active fractions were pooled, 3-fold concentrated by dialysis against column buffers containing 50% glycerol, and stored at −20 °C. In agreement with previous results (4), DNA ligase I preparations by this procedure were approximately 1000-fold purified in 10% yield and had specific activities of 0.01–0.02 unit (12)/mg protein, while DNA ligase II preparations were about 200-fold purified in 15% yield and had specific activities of 0.003–0.005 unit/mg protein. Phage T4 DNA ligase was purchased from New England Biolabs.

Ligase Assays—Reaction mixtures (40 μl) contained 70 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 50 μg ml^{-1} nuclease-free bovine serum albumin, polydeoxynucleotide substrates, or DNA, and a limiting amount of DNA ligase (1×10^{-7} units when not otherwise stated). Incubations were at 16 °C for 1 h. Using a poly(dA)·oligo(dT) substrate (3000 cpm), the assay was linearly dependent on enzyme concentrations within the range 0–1.5×10^{-7} enzyme units for either mammalian DNA ligase I or ligase II. Hybrid substrates were prepared by annealing 5′-^{32}P-labeled oligo(dT)$_{25-30}$ to poly(rA). Oligo(dT)·poly(dA) substrates were similarly prepared and used for enzyme assays during purification. The conversion of 5′-^{32}P-labeled phosphomonoesters to alkaline phosphatase-resistant diesters was measured (12, 13). Poly(dA), poly(rA), and oligo(dT)$_{25-30}$ were purchased from P-L Biochemicals. The oligo(dT) was treated with alkaline phosphatase and then radioactively labeled (in the presence of 2 mM K_2HPO_4 to inhibit the phosphatase) with [γ-^{32}P]ATP (3000 Ci mmol^{-1}) and T4 polynucleotide kinase.

Nicked circular DNA was prepared by *Eco*RI cleavage of plasmid pAT153 in the presence of ethidium bromide (14); 0.2 μg of nicked DNA was included in each 20-μl reaction mixture with 1.5×10^{-6} units (12) of T4 DNA ligase, or mammalian ligase I or II. Reactions were allowed to proceed for 1 h at 14 °C and were terminated by extraction with buffered phenol:chloroform (1:1). The DNA solutions were subsequently characterized by agarose gel electrophoresis.

Blunt-ended DNA substrates were prepared by cleavage of ϕX174 replicative form DNA with *Hpa*I. DNA (0.2 μg) was incubated in a

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40-μl standard reaction mixture supplemented with polyethylene glycol 6000 (17.5% when not otherwise stated) for 1 h at 37 °C with 1.5×10^{-6} units (when not otherwise stated) of T4 DNA ligase or mammalian DNA ligase I or II.

Determination of 3′-Mononucleotides—Poly(dA)·oligo([5′-^{32}P]dT) and poly(rA)·oligo([5′-^{32}P]dT), treated with 3×10^{-7} units of DNA ligase I or DNA ligase II to obtain maximum joining, were then treated with alkaline phosphatase to remove susceptible residues, heated at 100 °C for 30 min to inactivate the enzyme, dialyzed, and then degraded to 3′-mononucleotides by digestion with micrococcal nuclease (Worthington) and spleen phosphodiesterase (Sigma) (12). ^{32}P-Labeled dTMP residues were chromatographed on a Partisil Sax HPLC column (1.5 × 25 cm) using 10 mM potassium phosphate, pH 3.0, to separate 5′-dTMP (elution time 10.8 min) from 3′-dTMP (elution time 12.8 min). Authentic nucleotide markers were purchased from Sigma.

Inhibition of Ligases—Ligase I antibody (0.5 μg, purified from serum by precipitation with ammonium sulfate (4)) was preincubated for 5 min at 4 °C with T4 ligase or mammalian ligase I or II in reaction mixtures. The conversion of nicked circular DNA to a covalently closed form by mammalian ligase I was inhibited, while ligase II and T4 ligase were not affected (Fig. 1). Heat inactivation experiments with the mammalian ligases I and II were performed as described previously (3).

RESULTS AND DISCUSSION

Joining of Hybrid Substrate—T4 DNA ligase effectively joins oligodeoxyribonucleotides hydrogen-bonded to a complementary polyribonucleotide, while *Escherichia coli* DNA ligase is unable to catalyze this reaction (2). Similarly to the *E. coli* enzyme, the ATP-dependent mammalian DNA ligase I does not show detectable activity with a hybrid oligo(dT)·poly(rA) substrate (9), as also observed here (Fig. 2). Thus, reaction mixtures containing a concentration (1.5×10^{-6} units) of DNA ligase I 10 times higher than required for maximal joining of a poly(dA)·oligo(dT) substrate yielded no detectable joining (<5%) of an equivalent amount of the poly(rA)·oligo(dT) substrate. The lack of detectable activity of ligase I with the hybrid substrate was not due to degradation of this substrate by contaminating mammalian RNase H, since artificial mixtures of our ligase I preparation and small amounts of T4 ligase were as active as the T4 enzyme by itself (data not shown). In contrast to the results obtained with ligase I, the mammalian ligase II joined the hybrid substrate at about 50% of the rate observed with a nicked double-stranded polydeoxyribonucleotide (Fig. 2). The reaction of DNA ligase II with either substrate was directly

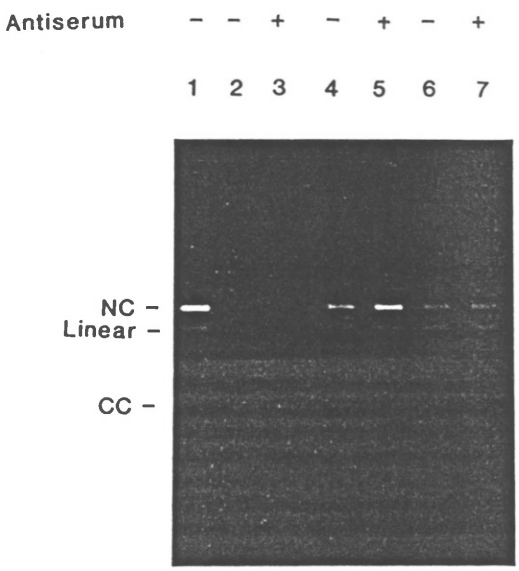

FIG. 1. **Activity of DNA ligases under standard assay conditions in the presence of antibodies against mammalian ligase I.** The conversion of nicked circular (*NC*) plasmid DNA to covalently closed molecules (*CC*) was visualized after electrophoretic separation on 1% agarose gels containing 0.5 μg ml^{-1} ethidium bromide. *Lane 1*, no ligase added; *lanes 2* and *3*, T4 DNA ligase; *lanes 4* and *5*, mammalian ligase I; *lanes 6* and *7*, mammalian ligase II. Ligase I antibody was included in reactions where indicated.

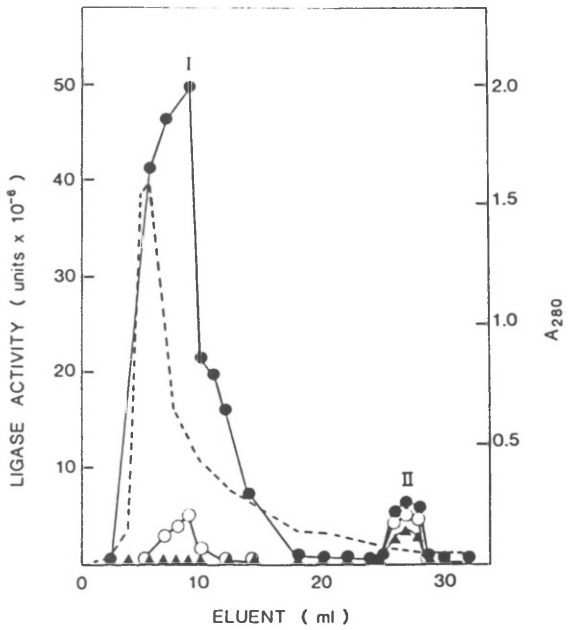

FIG. 2. **Assays of mammalian DNA ligases I and II with a hybrid substrate.** The two calf thymus DNA ligases (from 50 g of tissue, material partly purified by phosphocellulose chromatography) were separated by hydroxyapatite gradient chromatography (3) and column fractions (5-μl aliquots) were assayed directly with poly(dA)·oligo([5′-^{32}P]dT) (●) or poly(rA)·oligo(dT) (▲) substrates, as described under "Experimental Procedures." Reactions with the poly(dA)·oligo(dT) substrate were also performed in the presence of antibodies to ligase I (○). *Dashed line* represents A_{280}.

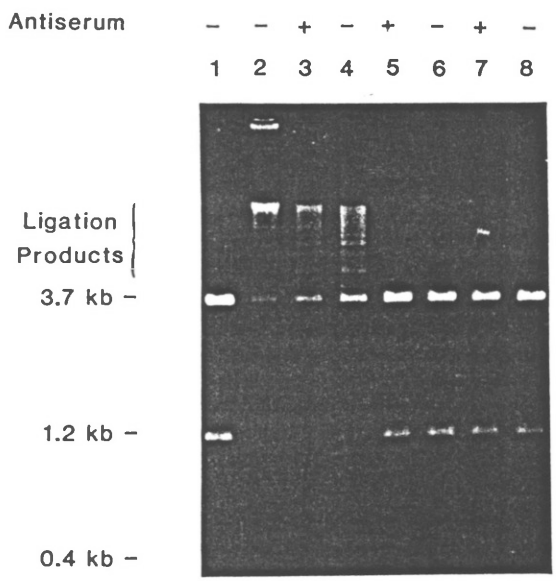

FIG. 3. **Blunt-end joining by DNA ligases.** The enzymes were assayed (as described under "Experimental Procedures") for their ability to join *Hpa*I fragments of φX174 replicative form DNA in the presence of 17.5% polyethylene glycol 6000. Reaction products were characterized by agarose gel electrophoresis as in Fig. 1. *Lanes 1* and *8*, no enzyme added; *lanes 2* and *3*, T4 DNA ligase; *lanes 4* and *5*, mammalian ligase I; *lanes 6* and *7*, mammalian ligase II. Antibody to ligase I was added as indicated.

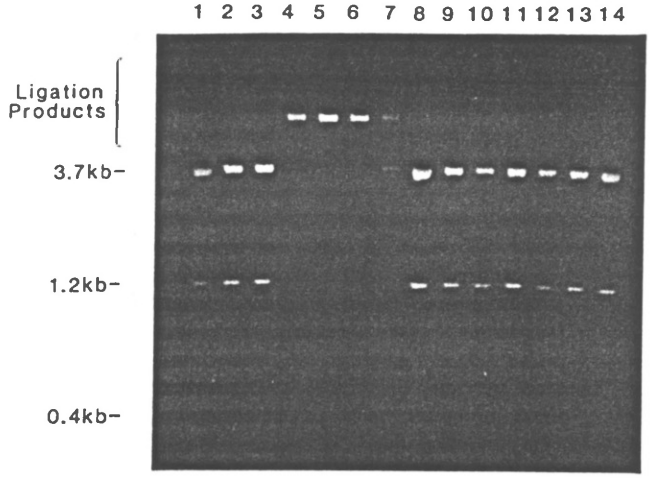

FIG. 4. **Blunt-end joining by DNA ligases at different concentrations of polyethylene glycol 6000.** Reactions were performed as described in Fig. 3. *Lanes 1* and *8*, no enzyme added; *Lanes 2–7*, mammalian ligase I and 0, 7.5, 12.5, 17.5, 20, 22.5% of polyethylene glycol, respectively; *Lanes 9–14*, mammalian ligase II and 0, 7.5, 12.5, 17.5, 20, and 22.5% of polyethylene glycol, respectively.

proportional to time (up to 2 h at 16 °C) and to enzyme concentration (up to 3×10^{-7} units). Moreover, the joining activities for oligo(dT) bound to either a poly(dA) or a poly(rA) complementary chain cochromatographed during hydroxyapatite chromatography (Fig. 2) as well as on further purification of ligase II by gel filtration. Furthermore, the two activities exhibited the same heat lability (50% inactivation in 5 min at 42 °C) and may be ascribed to the same enzyme. As shown earlier, ligase II is unable to join strand interruptions in double-stranded polyribonucleotides, or single-stranded oligo(dT) molecules (1). By comparison, ligase II must be more than 100-fold more active than ligase I with the poly(rA)·oligo(dT) substrate under our standard assay conditions and a range of related conditions. The presence of a distinct catalytic activity of mammalian ligase II, not found in ligase I, would appear to confirm that the two enzymes represent different gene products.

Formation of 3′-5′-Phosphodiester Bonds—In order to demonstrate that the alkaline phosphatase resistance of radioactive phosphate residues in the polymer substrates treated with mammalian DNA ligase I or ligase II was due to the formation of phosphodiester bonds, the substrates were degraded to mononucleotides with micrococcal nuclease and spleen phosphodiesterase (12). Generation of 3′-5′-phosphodiester bonds between oligo([5′-^{32}P]dT) moieties hydrogen-bonded to poly(dA) or poly(rA) would allow the recovery of [3′-^{32}P]dTMP, while this would not be possible if no ligation had occurred. After incubation of poly(dA)·oligo([5-^{32}P]dT) with DNA ligase I (1.5×10^{-7} units) under standard reaction conditions, more than 90% of the phosphatase-resistant radioactive material was recovered as 3′-dTMP after analysis by high pressure liquid chromatography. Similarly, incubation of poly(rA)·oligo([5′-^{32}P]dT) with mammalian DNA ligase II (3×10^{-7} units) allowed the isolation of >90% of the phosphatase-resistant radioactive material as 3′-dTMP. These data show that both ligase I and ligase II act as DNA ligases, and that ligase II generates phosphodiester bonds with the poly(rA)·oligo(dT) hybrid substrate.

Blunt-end Joining of DNA—The T4 DNA ligase can join blunt-ended DNA fragments (2), and this activity has often been employed for the construction of recombinant DNA molecules. This function is less efficient than the sealing of single-strand interruptions in DNA, but blunt-end joining can be promoted in reaction mixtures by macromolecular crowding conditions, *e.g.* by the addition of polyethylene glycol (15). When the mammalian DNA ligases I and II were assayed with a blunt-ended DNA substrate in the presence of 17.5% polyethylene glycol 6000 (Fig. 3), ligase I was able to perform this joining reaction (*lane 4*), while ligase II showed no detectable activity (*lane 6*). The activity of ligase I was blocked by antibodies against the enzyme (*lane 5*) while T4 DNA ligase was not similarly inhibited (*lanes 2* and *3*). Further, the activity of ligase I was proportional to enzyme concentration (up to 1.5×10^{-6} units) and approximately linearly dependent on time up to 60 min. Increasing the enzyme concentration 5-fold, decreasing the temperature to 16 °C while increasing the time of incubation, or supplementing the reaction mixture with KCl (50–200 mM) failed to reveal any detectable activity of ligase II with the blunt-ended DNA substrate.

To determine the efficiencies of blunt-end joining at different concentrations of polyethylene glycol 6000, the concentrations of this reagent were varied in reaction mixtures (Fig. 4). Mammalian DNA ligase I showed optimal blunt-end joining at 17.5% polyethylene glycol 6000 (*lane 5*), with lower but still detectable joining occurring at 12.5% (*lane 4*) and 22.5% (*lane 7*) polyethylene glycol. Blunt-end joining by ligase I was not observed after 1 h at 37 °C in the absence of added polyethylene glycol 6000 (*lane 2*) but could be detected after incubation at 16 °C for 72 h (~30% of maximal joining). We estimate that in the absence of polyethylene glycol, DNA ligase I performs blunt-end joining of DNA 20–50 times less efficiently than sealing of single-strand interruptions. No detectable blunt-end joining catalyzed by DNA ligase II was observed at any polyethylene glycol concentrations (*lanes 9–14*), either at 37 or 16 °C. We conclude that mammalian DNA ligase I, but not ligase II, is able to join blunt-ended DNA molecules under our standard assay conditions. Zimmerman and Pfeiffer (15) have reported that a partly purified DNA ligase preparation from rat liver nuclei could seal double-strand breaks in DNA, and this activity may now tentatively be assigned to DNA ligase I.

Ligase Heterogeneity—The number of mammalian DNA ligases has been a controversial matter. The existence of ligases I and II as two separate activities (4) has been confirmed by Creissen and Shall (16) and by Chan and Becker (6, 11). However, Teraoka and Tsukada (10) have reported that mammalian cells only seem to contain one DNA ligase, corresponding to ligase I, and these authors and Mezzina *et al.* (17) have further suggested that different size classes of mammalian ligase might perhaps be ascribed to proteolysis. In contrast, in a more recent publication (7), Teraoka and Tsukada have confirmed our finding that antibodies against calf thymus DNA ligase I do not inhibit ligase II. Yeast cells appear to have a single DNA ligase (18) but this does not necessarily imply a similar situation for mammalian cells; for example, DNA polymerase β has not been found in yeast, although it is widely distributed among higher eukaryotes (19). The availability of separate and specific assay procedures for each of the two mammalian DNA ligase activities, as described here, should help to further clarify the situation. Since only one of the two enzymes may be required for essential replication events, it becomes an interesting possibility that one of the inherited human syndromes associated with retarded strand joining of damaged DNA could be associated with a molecular defect in a DNA ligase (20, 21).

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DNA ligase I deficiency in Bloom's syndrome

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Certain rare human diseases with autosomal recessive mode of inheritance are associated with a greatly increased cancer frequency which may reflect specific defects in DNA repair or replication. These disorders include xeroderma pigmentosum, ataxia-telangiectasia, Fanconi's anaemia and Bloom's syndrome[1]. Cells from individuals with Bloom's syndrome[2] usually grow slowly in culture and exhibit increased chromosomal breakage and rearrangement, an elevated frequency of sister chromatid exchanges, retarded rates of progression of DNA replication forks, delayed conversion of replication intermediates to high-molecular-weight DNA, and slightly increased sensitivity to DNA-damaging agents[3-11]. Several of these features are also characteristic of *Escherichia coli* and yeast mutants with a defective DNA ligase[12-22]. In this investigation we show that one of the two DNA ligases of human cells, ligase I, is defective in a representative lymphoid cell line of Bloom's syndrome origin.

Mammalian cells, in contrast to bacteria and yeast, have two different DNA ligases, which we have designated ligase I and ligase II (refs 23-25). Both enzymes are present in the cell nuclei but they do not cross-react serologically[23-28]. The larger enzyme, DNA ligase I, is induced during cell proliferation and may be active in chromosomal replication, whereas the smaller enzyme, ligase II, is present in similar amounts in growing and non-growing cells. Recently, selective assay procedures have been devised for these enzymes to facilitate their characterization[29]. Thus, only ligase I can catalyse the joining of blunt-ended DNA fragments, whereas ligase II can link oligo(dT) molecules, annealed to poly(rA).

The levels of DNA ligases I and II were measured in human lymphoid B-cell lines (from the Human Genetic Mutant Cell Repository, Camden, New Jersey) representative of different syndromes. Extracts of the cells were size-fractionated by liquid chromatography and assayed for total DNA ligase activity using a poly(dA)·oligo 5'-^{32}P-(dT) substrate[29,30]. In control cell lines, a large peak of DNA ligase I of relative molecular mass 200,000 (M_r 200K) and a smaller peak of ligase II (M_r 80K) were observed. Typical data are shown in Fig. 1*a*. These peaks represent the active monomers[26,27] of DNA ligases I and II. Under these extraction and fractionation conditions, no dimers[28] of DNA ligase I were observed. That the second peak of activity was DNA ligase II was confirmed by employing a poly(rA)·oligo(dT) substrate[29] (Fig. 1*a*). Elution profiles not significantly different from those of control cells were seen with extracts from lines GM2250 (xeroderma pigmentosum, complementation group A) (Fig. 1*b*), GM4510 (Fanconi's anaemia, data not shown) and GM1526 (ataxia-telangiectasia) (Fig. 1*c*). We conclude that there is no general deficiency in DNA ligase activity in cells representative of human chromosome breakage syndromes.

When the Bloom's syndrome cell line GM3403 was investigated, a reduced amount of DNA ligase I activity was observed (Fig. 1*d*), whereas the level of DNA ligase II was similar to that seen with control cells. This lymphoid cell line was derived from a confirmed case of Bloom's syndrome of Ashkenazi origin by the Human Genetic Mutant Cell Repository in collaboration with Dr K. H. Kraemer (NCI); it has retained the properties of high sister chromatid exchange and chromosome instability characteristic of the disease. The residual activity eluting at the expected position of ligase I in the GM3403 line was inhibited by 80% using rabbit antibodies[23] against DNA ligase I (Fig. 1*d*), which confirms that this high M_r peak of DNA ligase represented a small amount of active DNA ligase I. Furthermore, this fraction showed significant ability to perform blunt-end ligation of DNA, when assayed as described[29]. Thus the GM3403

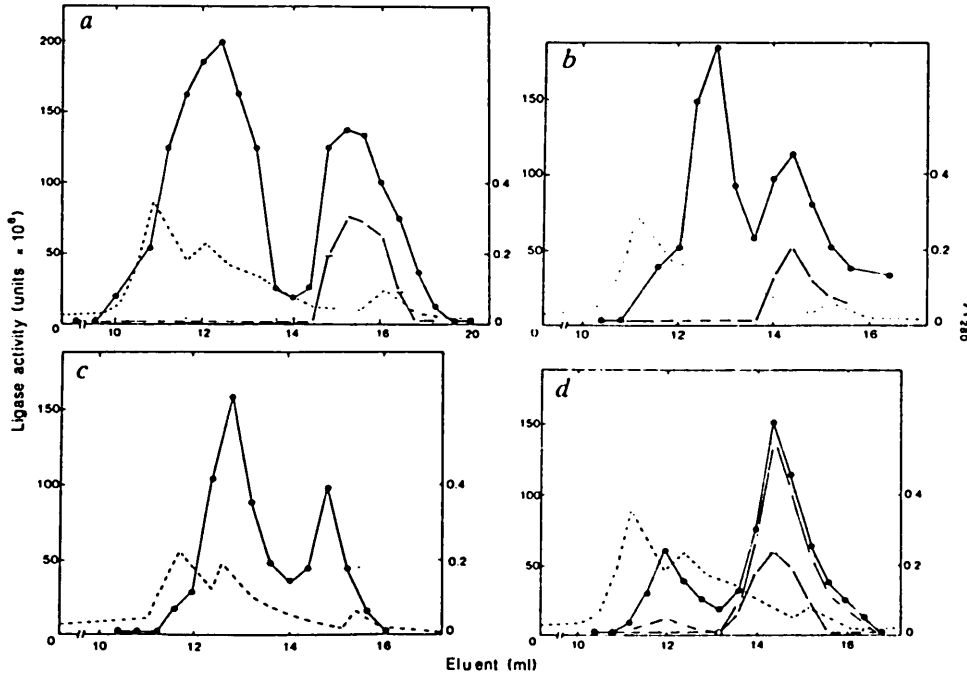

Fig. 1 DNA ligase activities in human lymphoid cell lines representative of different human inherited syndromes with possible DNA replication or repair defects. ●, Assays with poly(dA)·oligo(dT) substrate, measuring both DNA ligase I and ligase II; ○, the same assay carried out in the presence of antiserum against ligase I (0.25 μg $(NH_4)_2SO_4$-fractionated antiserum[23] per reaction mixture); △, results with a poly(rA)·oligo(dT) substrate, measuring specifically DNA ligase II (ref. 29); dashed line, A_{280}. The cell lines analysed were: *a*, Raji, a Burkitt's lymphoma line; *b*, GM2250, xeroderma pigmentosum, complementation group A; *c*, GM1526, ataxia-telangiectasia; *d*, GM3403, Bloom's syndrome.

Methods. Cells were grown in RPMI 1640 medium supplemented with 15% fetal calf serum and antibiotics. The cells (5×10^7-1×10^8) were collected by centrifugation, washed twice in phosphate-buffered saline, re-suspended in 300 μl ice-cold extraction buffer (0.1 M NaCl, 50 mM Tris-HCl, *p*H7.5, 10 mM 2-mercaptoethanol, 1 mM EDTA, 23 μg ml^{-1} aprotinin, 0.5 μg ml^{-1} each of pepstatin, leupeptin and chymostatin) and disrupted in a glass hand homogenizer. After 1 h at 0 °C, debris was removed by centrifugation for 10 min in an Eppendorf microfuge, and the crude cell extract supplemented with 0.05 volume each 2 M NaCl and 10% Polymin-P to precipitate nucleic acids. After 30 min at 0 °C, the material was centrifuged and the supernatant applied to an FPLC Superose-12 column (Pharmacia) equilibrated with 50 mM NaCl, 50 mM Tris-HCl (*p*H 7.5), 10 mM 2-mercaptoethanol, 1 mM EDTA. The column was eluted at 0.4 ml min^{-1} 200 μl fractions were collected, and DNA ligase assays were performed on 50 μl aliquots in a final reaction mixture volume of 60 μl.

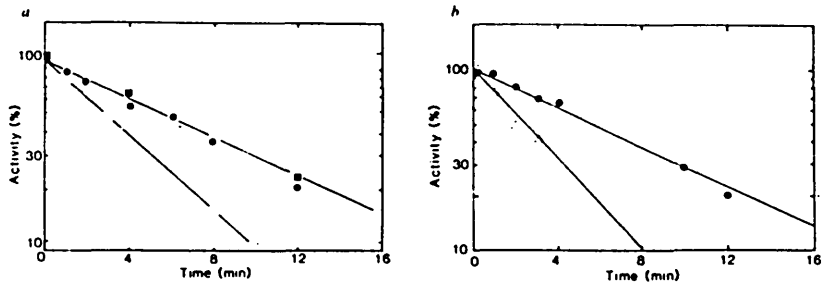

Fig. 2 Heat lability of DNA ligase I from a Bloom's syndrome cell line at different stages of enzyme purification. *a*, Pooled peak fractions (0.1–0.2 mg protein per ml) of the size-fractionated DNA ligase I (Fig. 1) were incubated at 50 °C, and aliquots removed at different times for ligase assays. ●, Ligase I from Raji cells; ■, ligase I from xeroderma pigmentosum group A; △, ligase I from Bloom's syndrome. *b*, In separate 10-fold scaled-up experiments, the Bloom's syndrome GM3403 line was propagated as 21 suspension cultures in 31 spinner vessels under conditions that allowed a growth rate similar to that observed for cell lines representative of other human syndromes: ●, △, as in *a*. DNA ligase I from Raji and GM3403 cells was purified by gel filtration on AcA-34 columns (LKB, Inc.) to obtain results similar to those shown in Fig. 1*a* and *d*. Pooled peaks of DNA ligase I activity were dialysed separately against 50 mM Tris-HCl (*p*H7.5)/5 mM KCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, and applied to columns of double-stranded DNA-cellulose[43]. These columns were eluted stepwise with the same buffer containing 50, 100 and 200 mM KCl. DNA ligase I was eluted at 100 mM KCl in both cases, and the enzyme was ~20-fold further purified by this procedure. The pooled active fractions of DNA ligase I (15 μg protein per ml) were incubated at 50 °C and assayed for ligase activity as in *a*.

cells, although not totally deficient in ligase I, contain one quarter of the activity found in other lymphoid lines.

The reduction in DNA ligase I activity in the Bloom's syndrome line could be due to a regulatory mutation, or to a structural defect in the enzyme itself. To distinguish between these possibilities, we determined the heat lability of the partly purified, size-fractionated DNA ligase I from the GM3403 line as well as from control cell lines (GM2250 and Raji). Figure 2*a* shows that the Bloom's syndrome line contains a DNA ligase I that is more heat-labile than the enzyme from control cells. On further purification of ligase I from the lines GM3403 and Raji by DNA-cellulose chromatography, the relative heat-sensitivity of the Bloom's syndrome enzyme persisted in comparison with the control enzyme (Fig. 2*b*). In contrast, DNA ligase II from the Bloom's syndrome cells showed the same heat lability (50% inactivation in 5 min at 42 °C) as ligase II from control cells (data not shown).

The GM3403 cell line is the only representative Bloom's syndrome lymphoid line available from a mutant cell culture collection: experiments with the fibroblast line GM5289, derived from a Japanese case of Bloom's syndrome, showed that DNA ligase I from this source was not obviously heat-sensitive or present in reduced amounts. However, in contrast to the enzyme from lymphoid cells (Fig. 1) or from HeLa cells, the GM5289 DNA ligase I eluted as a mixture of 30% dimers[28] and 70% monomers on size fractionation by liquid chromatography (data not shown). Altered aggregation properties of DNA ligase I in Bloom's syndrome cells have also been observed by Chan *et al.*[31].

Certain cell lines derived from Bloom's syndrome appear to represent revertants that no longer exhibit high sister chromatid exchange or other typical features of cells from patients[32,33]. For example cells from the lymphoid cell line GM4408 (Human Genetic Cell Repository) do not show elevated sister chromatid exchange and we have found normal levels of both DNA ligases I and II in cell extracts (data not shown). An interesting example of a strain with altered properties is the fibroblast line GM1492, which differs from other Bloom's syndrome fibroblasts in growing well in culture and having a preneoplastic phenotype[34]; these cells contain high levels of a presumably recombinogenic protein similar to the *E. coli* RecA protein[35] and a factor which appears to promote DNA ligase activity (S. Ljungquist, personal communication).

There is no evidence for multiple genetic complementation groups in Bloom's syndrome, and this inherited disease may well be due to a single defective polypeptide. Our results suggest that this protein is identical to DNA ligase I. Note that DNA ligase II (this work) as well as DNA polymerases α, β and γ (refs 36, 37) are normal in Bloom's syndrome cells. A number of different possible explanations for the cellular defect in Bloom's syndrome have been advanced previously, such as the release of a clastogenic factor or altered regulation of the synthesis of DNA repair enzymes[38–42], but these theories do not seem to account for the observed phenotypic changes. In contrast, a DNA ligase deficiency could explain the increased frequency of sister chromatid exchanges, because retarded joining of DNA strand interruptions would be expected to promote homologous recombination events. Furthermore, a defective DNA ligase could account for the anomalously slow conversion of DNA replication intermediates to a high M_r form. Because ligase I is likely to be required for DNA replication, presumably only leaky mutants can survive, and the variable phenotype of different cellular strains is probably related to different amounts of residual enzyme activity. The apparent relationship of Bloom's syndrome to an alteration in the structural gene for DNA ligase I makes the molecular cloning of this gene a priority, and suggests experimental strategies for the detection of aberrant DNA sequences in heterozygotes.

Since submission of this manuscript, a second Bloom's syndrome lymphoblastoid cell line (obtained from Dr E. Henderson, Temple University School of Medicine, Philadelphia) has also been found to contain reduced amounts of ligase I, and the enzyme is likewise anomalously heat-sensitive.

We thank Sally Tomlinson for her help with growing Bloom's syndrome cells, and Drs Mark Meuth and Dana Lasko for critical readings of the manuscript.

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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 (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, Gianelli *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 Wilm 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. 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 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 (●●●●●●) 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. They seemed 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

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

control cells, enzyme assays revealed a major peak of the 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 lines 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. An 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

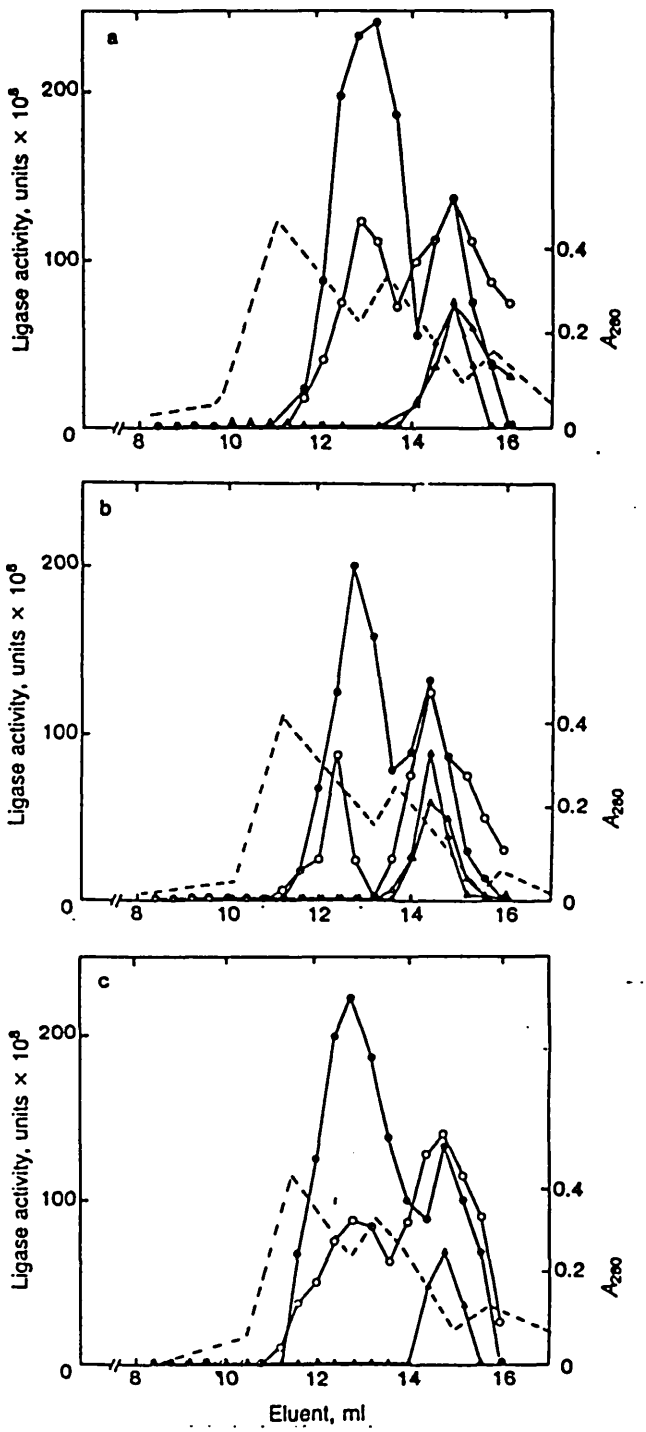

FIG. 1. Size fractionation of DNA ligase activities in representative BS cell lines. FPLC Superose-12 column profiles of Polymin P-treated cell extracts are shown. (*a*) Lymphoid cell lines W67-4 (Ashkenazi BS) and AG3829 (Werner syndrome). (*b*) Simian virus 40-transformed fibroblast line GM8505 (Ashkenazi 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.

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

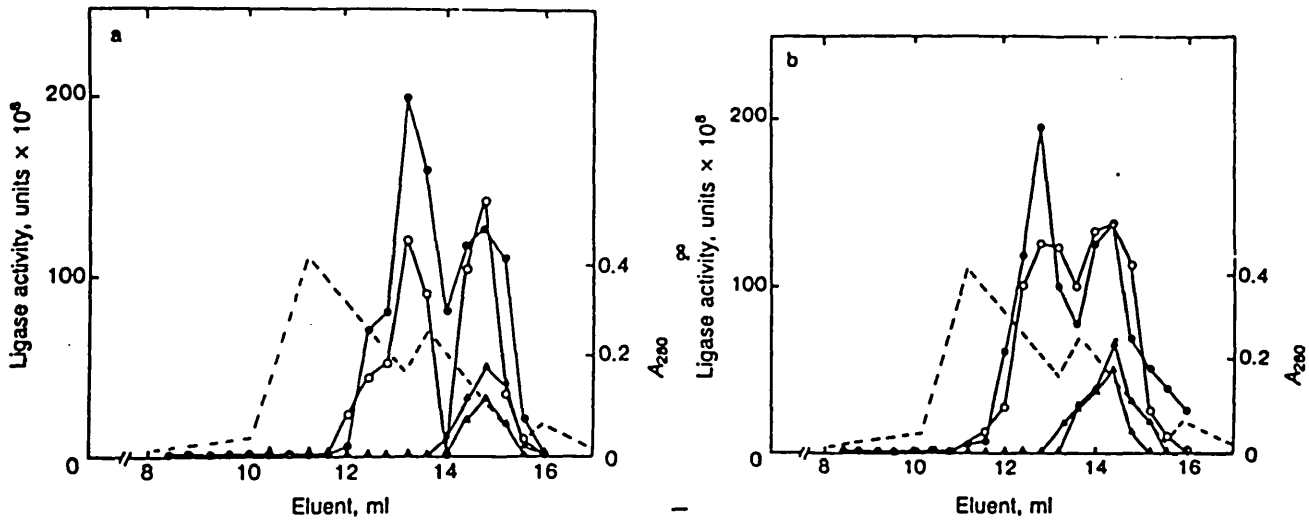

FIG. 2. DNA ligase activities in BS lymphoid cell lines with low SCE and in a heterozygote line. Chromatographic conditions and symbols are as in Fig. 1. (*a*) Lines D86-1-2 (Ashkenazi BS) and PS (Friedreich ataxia); (*b*) Lines AA87-5-1 (Ashkenazi BS) and AA87-4 (heterozygote; parent of patient from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

data not shown). These results confirm that the increased 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 approximately 70% reduced activity and 2-fold increased thermal inactivation 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 (R. W., unpublished data), 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

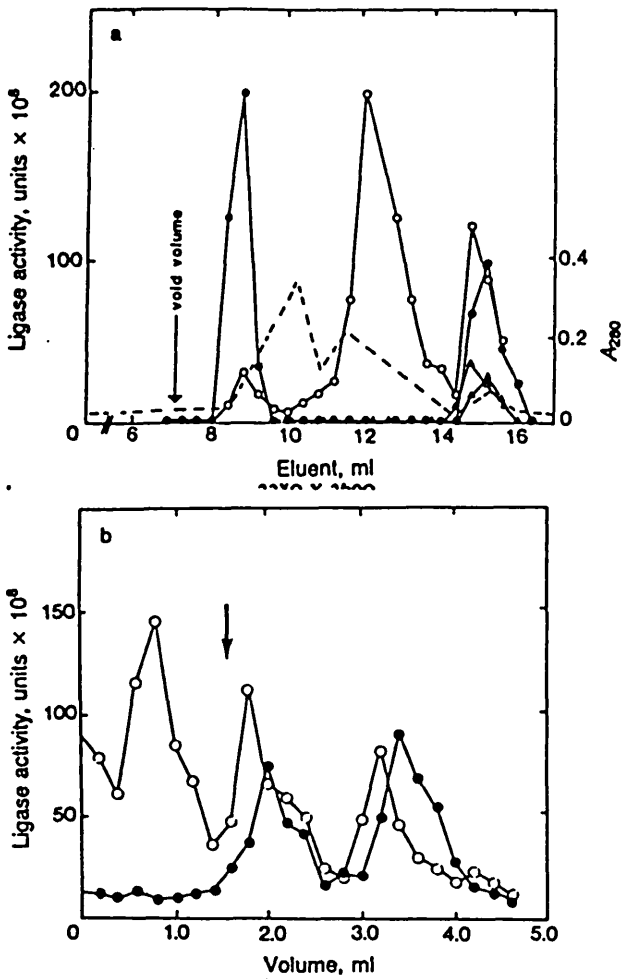

FIG. 3. DNA ligase activities in the Anglo-Saxon BS line 1032, containing a dimeric form of ligase I. (*a*) FPLC profiles as in Fig. 1 for closed symbols. The open symbols show the same experiment, but with incubation of the cell extract in 1 M NaCl prior to chromatography. (*b*) Sucrose gradient centrifugation. 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.

cells and showed an apparent 20% reduction in molecular 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

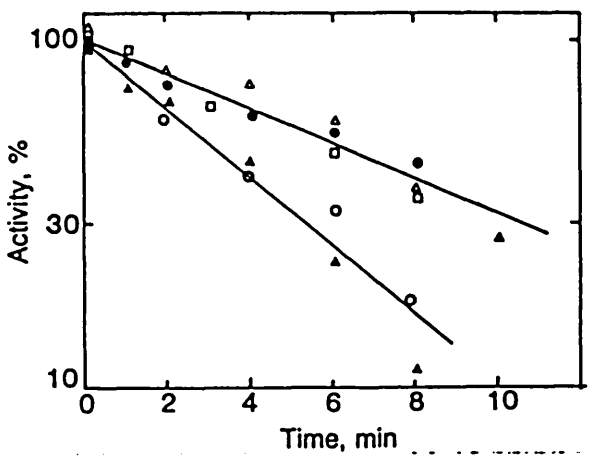

FIG. 4. Heat lability of DNA ligase I in BS. Ligase I peak fractions from FPLC were incubated at 50°C, and aliquots were removed at various times and assayed as described (15). The symbols indicate material from Ashkenazi BS line W67-4 (○), Mennonite BS line 1004 (▲), Anglo-Saxon BS line 1032 (□), BS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

SCE in our biochemical experiments. It seems likely that a 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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