The EMBO Journal vol.8 no.5 pp.1419-1425, 1989

Joining of linear plasmid DNA is reduced and error-prone in Bloom's syndrome cells

Thomas M.Rünger and Kenneth H.Kraemer

Laboratory of Molecular Carcinogenesis, National Cancer Institute, Bethesda, MD 20892, USA

Communicated by T.Lindahl

A linearized, replicating, shuttle vector plasmid, pZ189, was used to measure *in vivo* DNA joining ability of cells from patients with the cancer-prone, immunodeficient, chromosome breakage disorder, Bloom's syndrome (BS). The BS cell lines we studied were reported to contain reduced *in vitro* activity of DNA ligase I. We assessed *in vivo* joining ability by transfecting linear plasmids with overlapping or blunt ends (produced by *Eco*RI or *Stu*I) into BS and normal fibroblast or lymphoblast host cells and measuring the amount of re-joined, replicated plasmids by their ability to transform bacteria. With plasmids having either overlapping or blunt ends we found a 1.3- to 3-fold lower ($P < 0.05$) joining efficiency in BS cells than in the normal cells. The mutation frequency of the recovered plasmids was measured by screening for function of the suppressor tRNA contained in pZ189, for plasmid size, for presence of restriction sites, or by DNA sequencing. The spontaneous mutation frequency with the circular plasmid was 0.05-0.08% with both BS cell lines, values 2- to 21-fold higher ($P < 0.03$) than with the normal cell lines. The mutation frequency with the linear plasmid passaged through both BS cell lines was 21-52%, values 1.4- to 5.4-fold higher ($P < 0.001$) than with the normal lines. Detailed analysis of 210 recovered plasmids revealed an increase ($P \leq 0.001$) in deletions, insertions or complex mutations at the joining sites, and in point mutations with the *Eco*RI cut plasmid with the BS cells in comparison to the normal cells. These data support the hypothesis of an *in vivo* DNA ligase deficiency in BS and in addition show an error-prone DNA end-joining process and spontaneous hypermutability. These abnormalities might be related to the increased chromosome breakage and immune dysfunction in BS patients.

Key words: Bloom's syndrome/DNA ligase/mutagenesis/immunodeficiency/DNA sequencing

Introduction

Bloom's syndrome (BS) is a rare autosomal recessive disease with a high cancer risk, immunodeficiency, growth retardation and telangiectatic facial skin lesions (Hütteroth *et al.*, 1975; German *et al.*, 1983, 1984; Gretzula *et al.*, 1987). Cells from patients with BS are characterized by an elevated level of spontaneous sister chromatid exchanges (SCE), chromosome breakage, spontaneous hypermutability (Warren *et al.*, 1981; Vijayalaxmi *et al.*, 1983; Langlois *et al.*, 1989), a higher level of recombinogenic activity (Bubley and Schnipper, 1987) and a DNA ligase I deficiency *in vitro* (Chan *et al.*, 1987; Willis and Lindahl, 1987).

In order to study DNA ligase activity *in vivo*, we developed a host cell ligation (end-joining) assay analogous to plasmid DNA repair host cell reactivation assays (Protić-Sabljić *et al.*, 1985; Bredberg *et al.*, 1986; Seetharam *et al.*, 1987; Seidman *et al.*, 1987), using the replicating shuttle vector plasmid pZ189 (Seidman *et al.*, 1985). Linear plasmid DNA was produced by digestion with restriction endonucleases and then transfected into fibroblast or lymphoblast host cells. After 2-3 days the plasmid was harvested and unreplicated input plasmid was digested with *Dpn*I, which cleaves DNA with the methylation pattern specific for DNA replicated in bacteria. Replicated plasmid was used to transform bacteria and plated onto selective agar dishes. Since only circular plasmids are able to replicate, each bacterial colony reflected end-joining that occurred during passage through the human cell. Scoring of bacterial colonies allowed a quantification of the *in vivo* host cell DNA end-joining process.

A mutagenesis marker gene in pZ189 allows screening for fidelity of the end-joining process. Measuring plasmid size, restoration of restriction sites and sequence analysis also provides information as to the quality of the end-joining process. Presence of the restriction site reflects faithful end-joining, while a loss indicates an alteration of the joining site.

We found a reduced *in vivo* end-joining of linear plasmids by BS cells. The joining in BS cells was error-prone with more modifications at the joining site and a higher rate of point mutations. In addition, circular plasmids showed an elevated spontaneous mutation frequency in BS cells.

Results

Plasmid survival

Transformation of bacteria with plasmid recovered after passage of circular plasmid through fibroblast and lymphoblast cell lines yielded a large number of bacterial colonies, demonstrating that pZ189 was able to replicate in both types of cells. The yield of bacterial colonies was 1.9- to 3.6-times lower after passage of the circular plasmid through BS cell lines than through normal cell lines ($P < 0.001$): the means were 1879 colonies/transformation in normal fibroblasts, 524 in BS fibroblasts, 14 856 in normal lymphoblasts and 7978 in BS lymphoblasts.

Southern blots of plasmid purified after passage of linear plasmids through normal fibroblasts demonstrated the presence of supercoiled and relaxed circular forms, indicating that these cells have the ability to join the ends of the linear plasmid *in vivo* (data not shown). No bands could be detected with linear plasmids passed through BS fibroblasts.

The yield of bacterial colonies formed after passage of linear plasmid through the normal and BS cells was 5- to

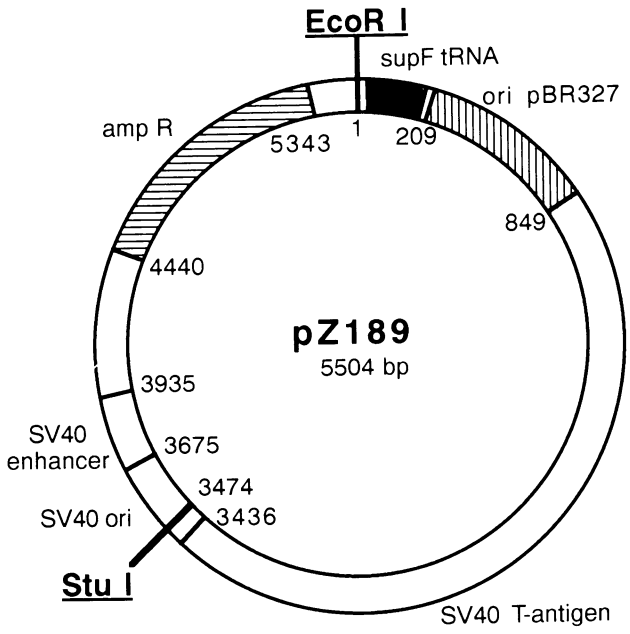

Fig. 1. Plasmid shuttle vector pZ189 used for host cell ligation (end-joining) assays. The *Eco*RI restriction site at bp 1 is located between the ampicillin resistance gene and the bacterial origin of replication from pBR327. Both genes are essential for plasmid survival in bacteria grown in the presence of ampicillin. The *Eco*RI site is near to the *supF* gene, which is used as a mutagenesis marker for point mutations and deletions. The *Stu*I site (bp 3473) lies within the SV40 sequences essential for survival of the plasmid in mammalian cells.

200-fold lower than with the circular plasmid ($P = 0.04$ for linear plasmids in BS fibroblasts, $P \leq 0.001$ for BS lymphoblasts and for the normal cell lines) (Figure 2). Since replicated plasmids must go through a circular stage, these colonies reflect joining that occurred during the passage through the host cells. The lower yield with the linear plasmids compared to the circular controls indicates that only a portion of the transfected linear plasmids was circularized.

The relative number of colonies after passage of linear plasmids through normal and BS cell lines was lower with the BS cells than with the normal cells for both blunt and overlapping ended plasmids (Figure 2). In BS fibroblasts the survival of linear plasmids with overlapping ends and with blunt ends was about 3-fold lower than in normal fibroblasts ($P = 0.03$), indicating a 3-fold lower *in vivo* joining efficiency in BS fibroblasts. The 1.4- to 1.7-fold difference in lymphoblasts was significant when comparing the combined joining efficiency of BS lymphoblasts with normal lymphoblasts ($P < 0.05$), but not significant separately for overlapping and blund ended plasmids.

In all four cell lines the survival of linear plasmids with blunt ends was lower than with overlapping ends (5.9 times in normal fibroblasts, $P = 0.02$; 7.0 times in BS fibroblasts, $P = 0.05$; 2.1 times in normal lymphoblasts, $P = 0.01$; 2.7 times in BS lymphoblasts, not significant). This indicates that *in vivo* joining of linear plasmids with blunt ends was less efficient than joining of plasmids with overlapping ends.

Classification of mutant plasmids

Sequence analysis of 53 blue, light blue and white plasmids recovered from BS and normal fibroblasts found a wide range of deletions (3−371 bp), point mutations in the *supF* gene (single and multiple), reverse insertions of plasmid

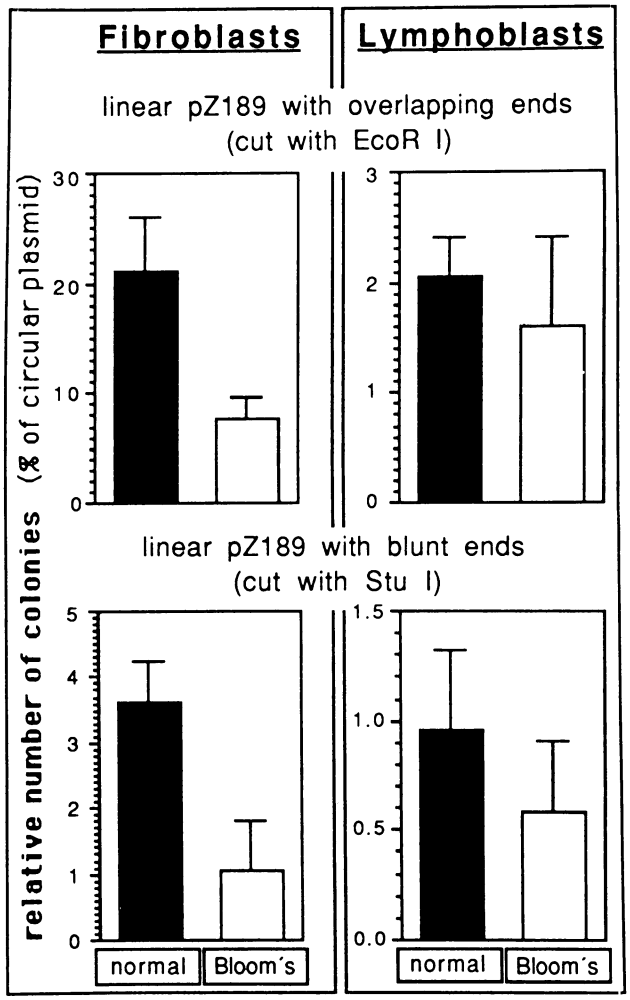

Fig. 2. Survival of linear plasmid pZ189 in either blunt or overlapping ends after passage through normal or Bloom's syndrome cell lines and transformation of *E.coli*. Numbers of bacterial colonies reflect joining efficiency to circular plasmid by the host cells. The survival of linear plasmids was calculated separately for each independent sample (10 samples in fibroblast and 4−5 in lymphoblasts with *Eco*RI cut plasmids; 4−6 samples with *Stu*I cut plasmids) as bacterial colony counts relative to the colony counts of a parallel control sample with untreated, circular plasmid and then averaged. The one-sided sign-test was used to test significance of differences in the overall plasmid survival and Student's *t*-test was used to test for significance of differences in the plasmid survival of plasmids with overlapping and blunt ends separately. The standard error of the mean (SEM) is shown in error bars.

DNA (12−>200 bp), insertions of non-plasmid DNA (58−>200 bp) and filling in of the overlapping ends at the *Eco*RI site. An ambiguity in the precise location of some deletions was due to short sequence junctional homologies ranging from 1 to 10 bp (three out of 10 deletions at the *Eco*RI site in BS fibroblasts, and four out of nine in normal fibroblasts). The color of the bacterial colony generated by a mutant with a deletion at the *Eco*RI restriction site made possible a differentiation into small and large deletions: deletions less than ~110 bp were too small to inactivate the *supF* gene and resulted in blue colonies. Deletions larger than ~215 bp at least partially inactivated the *supF* gene, resulting in light blue or white colonies. The location of the *supF* gene between the ampicillin resistance gene, starting at bp 5343, and the pBR327 *ori* gene, starting at bp 209 (Figure 1),

Table I. Criteria for classification of mutants of the plasmid pZ189 without sequencing

Input plasmid	Color of bacterial colony	Plasmid size (compared to wild-type)	Conservation of restriction site[a]	Classification
Circular, *Eco*RI cut linear or *Stu*I cut linear	blue	same	+	wild-type
		same smaller	−	(small) deletion
		larger	+ −	insertion[b] or more complex mutation[c]
Circular or *Eco*RI cut linear	white light blue	same	+	point mutation
		same smaller	−	(large) deletion
		larger	+ −	insertion[b] or more complex mutation[c]
*Stu*I cut linear	white light blue	same	+	point mutation
		same	−	insertion[b] or more complex mutation[c]
		smaller larger	+ −	insertion[b] or more complex mutation[c]

[a]+, restriction site is conserved; −, restriction site is lost; + −, restriction site may be either conserved or lost.
[b]Insertion at joining site.
[c]Deletions or insertions elsewhere in the plasmid or a combination of deletions or insertions plus point mutations.

limited the size of the possible deletions to less than ~400 bp, because these two genes are essential for plasmid survival. The largest deletion found was 371 bp. The largest deletion at the *Stu*I site found with the *Stu*I linearized plasmid was 18 bp. This site is located in a region essential for replication in mammalian cells (the SV40 origin) and larger deletions would inactivate the plasmid. Point mutations can only be detected when they at least partially inactivate the *supF* marker gene. Point mutations elsewhere in the plasmid are not detectable.

Based on these sequencing results mutants were classified into three categories, depending on color of the bacterial colony, size of the mutant plasmid compared to the wild-type size and restoration of the restriction site at which the plasmid was linearized (Table I). These categories were: (i) deletions at the joining sites, (ii) insertions at the joining sites or more complex mutations (deletions or insertions elsewhere in the plasmid or a combination of deletions or insertions plus point mutations) and (iii) point mutations. These criteria allow classification of mutants without sequencing, but do not permit differentiation between insertions and more complex mutations.

In order to test these criteria, the results of the sequence analysis of the 53 mutants was compared to their classification using the non-sequencing criteria. Of the sequenced mutants, 96% (51 out of 53) would have been classified correctly with these criteria. The two exceptions were mutants with a small insertion or a complex mutation, that were classified incorrectly as deletions or point mutations.

Frequencies and spectrum of mutations

With BS cell lines the spontaneous mutation frequencies with the circular plasmid were 2.2- to 20.8-fold higher than with normal cell lines ($P \leq 0.03$): mutations were 16 out of 73 274 colonies (= 0.022 ± 0.005%) in normal fibroblasts, 15 out of 31 781 colonies (= 0.047 ± 0.012%) in BS fibroblasts, 11 out of 297 109 colonies (= 0.004 ± 0.001%) in normal lymphoblasts and 65 out of 84 657 colonies (= 0.077 ± 0.0095%) in BS lymphoblasts.

When transfected wth linear plasmids, total mutation frequencies were 250- to 25 000-times higher than the spontaneous mutation frequencies with the circular plasmid ($P < 0.001$) (Figure 3). The plasmids passed through BS cells showed a 1.4- to 5.4-fold higher ($P \leq 0.001$) mutation frequency compared to plasmids passed through normal cells.

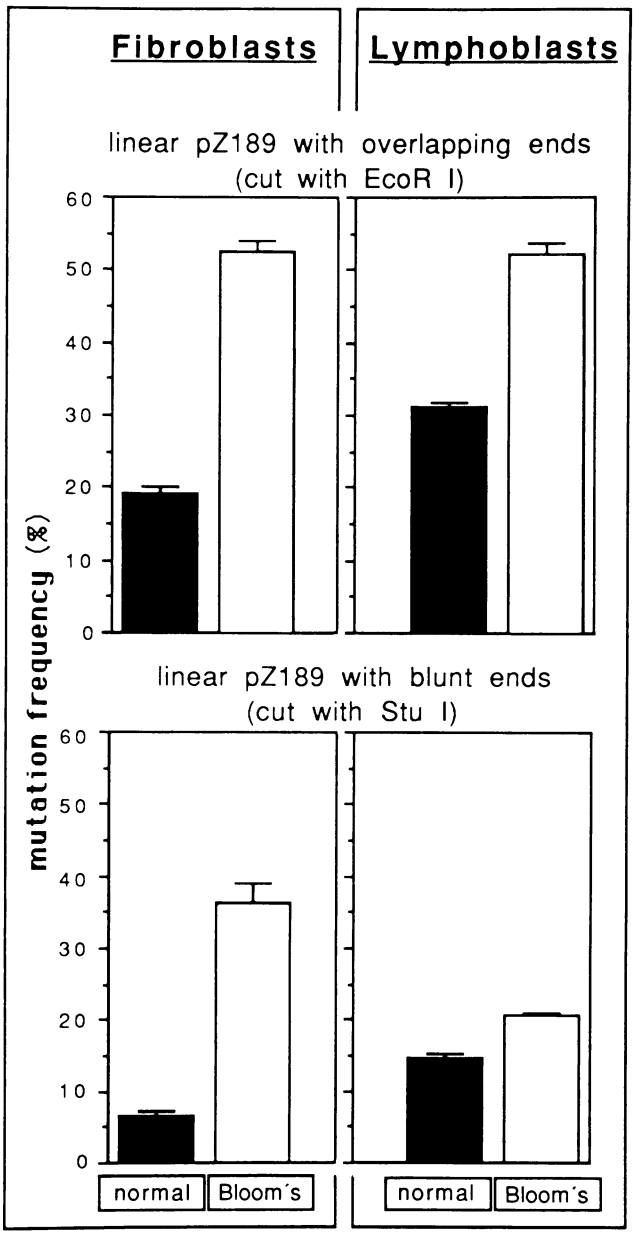

Fig. 3. Total mutation frequencies of linear plasmid pZ189 with either blunt or overlapping ends joined during passage through normal or BS cell lines. SEM of the mean is shown in error bars.

Table II. Frequency and types of mutations obtained after passage of linear plasmid pZ189 through Bloom's syndrome or normal cell lines

	Normal (%)	Bloom's syndrome (%)	Bloom's/normal
Linear plasmid with overlapping ends (cut with EcoRI)			
Fibroblasts:			
deletions at the *Eco*RI site	11.0 ± 0.1[a]	35.6 ± 0.1	3.2 $P < 0.001$
insertions at the *Eco*RI site or more complex mutations	8.3 ± 0.6	12.9 ± 0.3	1.6 $P < 0.001$
point mutations	0.0 ± 0.0	3.9 ± 1.1	– $P = 0.001$
total mutation frequency	19.3 ± 0.7	52.2 ± 1.5	2.7 $P < 0.001$
no. of analyzed plasmids (no. of independent samples)	73 (10)	30 (10)	
Lymphoblasts:			
deletions at the *Eco*RI site	23.6 ± 0.4	30.7 ± 0.6	1.3 $P < 0.001$
insertions at the *Eco*RI site or more complex mutations	7.7 ± 0.1	17.5 ± 0.6	2.3 $P < 0.001$
point mutations	0.0 ± 0.0	4.0 ± 0.3	– $P < 0.001$
total mutation frequency	31.3 ± 0.5	52.2 ± 1.5	1.7 $P < 0.001$
no. of analyzed plasmids (no. of independent samples)	54 (5)	53 (4)	
Linear plasmids with blunt ends (cut with StuI)			
Fibroblasts:			
deletions at the *Stu*I site	0.0 ± 0.0	19.0 ± 4.4	– $P = 0.001$
insertions at the *Stu*I site or more complex mutations	6.3 ± 0.5	7.6 ± 1.8	1.2 NS
point mutations	0.4 ± 0.3	9.8 ± 3.5	24.5 $P = 0.010$
total mutation frequency	6.7 ± 0.5	36.4 ± 2.6	5.4 $P < 0.001$
no. of analyzed plasmids (no. of independent samples)	20 (6)	28 (5)	
Lymphoblasts:			
deletions at *Stu*I site	10.2 ± 0.1	16.5 ± 0.1	1.6 $P < 0.001$
insertions at *Stu*I or more complex mutations	3.7 ± 0.2	4.2 ± 0.1	1.1 NS
point mutations	0.8 ± 0.6	0.02 ± 0.02	0.025 NS
total mutation frequency	14.7 ± 0.7	20.7 ± 0.1	1.4 $P = 0.001$
no. of analyzed plasmids (no. of independent samples)	37 (5)	38 (4)	

[a]Mean ± SEM. The mutation frequency was calculated separately for the blue and the light blue or white colonies, added for each independent sample and averaged. The mutation frequency for blue colonies was assessed by the analysis of plasmids purified from blue colonies as ratio: number of mutated plasmids/total number of analyzed plasmids, and calculated for each independent sample. The frequency of a particular class of mutation was determined by multiplying the mutation frequency of blue colonies of each independent sample with the frequency of this class of mutation in a representative sample of blue colonies. The sums of this rate in blue colonies and the similarly calculated rate in light blue or white colonies was then averaged. Student's *t*-test was performed to test for significance. NS = not significant.

Table III. Frequency and types of mutations obtained after passage of circular plasmid pZ189 through Bloom's syndrome or normal cell lines

	Normal (%)	Bloom's syndrome (%)	Bloom's/normal
Fibroblasts:			
deletions at the joining site	0.004 ± 0.002[a]	0.0 ± 0.0	–NS
insertions at the joining site or more complex mutations	0.0 ± 0.0	0.016 ± 0.007	–NS
point mutations	0.018 ± 0.005	0.031 ± 0.01	1.7 NS
total mutation frequency	0.022 ± 0.005	0.047 ± 0.012	2.2 $P = 0.03$
no. of analyzed plasmids (no. of independent samples)	12 (15)	8 (28)	
Lymphoblasts:			
insertions at the joining site or more complex mutations	0.0 ± 0.0	0.032 ± 0.0062	– $P < 0.001$
point mutations	0.004 ± 0.001	0.045 ± 0.0073	11.3 $P = 0.004$
total mutation frequency	0.004 ± 0.001	0.077 ± 0.0095	20.8 $P < 0.001$
no. of analyzed plasmids (no. of independent samples)	24 (8)	28 (5)	

[a]Mean ± Poisson standard error. The baseline mutation frequency with the circular plasmid was calculated as number of light blue or white colonies/total number of colonies. The Fisher's exact test was used to test for significance. NS = not significant.

Table II shows the detailed characterization of 333 plasmids recovered from samples with linear input plasmids. Significantly higher mutation frequencies were found with BS cells for deletions at the joining sites with either *Eco*RI cut or *Stu*I cut plasmids ($P \leq 0.001$) in both types of cells, for insertions at the joining site or more complex mutations, and point mutations with the *Eco*RI cut plasmids ($P \leq 0.001$) in both types of cells, and for point mutations with *Stu*I cut plasmids in fibroblasts ($P = 0.01$). Of all deletions at the *Eco*RI restriction site, 73.4% were small in normal

fibroblasts, 88.8% in BS fibroblasts, 60.1% in normal lymphoblasts and 55.2% in BS lymphoblasts, suggesting no difference in the size distribution of deletions between BS and normal cells.

In order to assess spontaneous alterations of the *Eco*RI restriction site, 38 plasmids from blue colonies obtained with circular plasmids passed through BS and normal cells, and 40 putative wild-type plasmids from originally *Stu*I cut samples were screened for conservation of the *Eco*RI restriction site: no spontaneous alteration of the *Eco*RI site was found. No spontaneous alteration of the *Stu*I restriction site was found in 12 plasmids from the blue control colonies (data not shown).

Table III shows the detailed characterization of 72 plasmids recovered from samples with circular input plasmids. In contrast to the normal cells, where no insertions or more complex mutations were found, more than one-third of the mutants recovered from BS cells were of this type. The frequency of spontaneous point mutations was 1.7- to 11.3-fold higher with BS cells. With the *Eco*RI cut linear plasmids in BS cells the frequency of point mutations increased ~100-fold ($P < 0.001$) compared to the controls transfected with the circular plasmid (Table III). This increase was not seen with the normal cell lines.

Discussion

The data indicate a reduced efficiency of *in vivo* joining of linear plasmid in BS fibroblasts and lymphoblasts. Two mammalian DNA ligases have been identified (Arrand *et al.*, 1986; Söderhäll and Lindahl, 1976): DNA ligase I is induced during cell proliferation, while DNA ligase II is present at a constant level throughout the cell cycle. DNA ligase I and II join single strand breaks in double-stranded DNA and DNA double strand breaks with overlapping ends. DNA ligase I is unique in its ability to ligate DNA with blunt ends. The BS cell lines used in our assay were reported previously to have a reduced *in vitro* ligase I activity of about one-third of normal with an abnormal heat lability (Willis *et al.*, 1987). Our findings of reduced joining of linear plasmid with blunt ends *in vivo* in BS cells is consistent with these *in vitro* results. Reduced joining of linear plasmid with overlapping ends is also consistent with a DNA ligase I deficiency, since double-stranded DNA with overlapping ends is ligated by DNA ligase II and by ligase I. In fibroblasts the extent of joining remaining (29–36%, Figure 2) measured with our assay was similar to the *in vitro* results (Willis *et al.*, 1987), whereas with lymphoblasts our assay showed a smaller difference (60–77% remaining, Figure 2). This may be related to the different transfection procedures used with fibroblasts and lymphoblasts.

These results with the replicating plasmid, pZ189, differ from our previous results with a non-replicating plasmid (Rünger and Kraemer, 1988), where we measured chloramphenicol acetyl transferase (CAT) activity in these same cells after introducing linear pRSV*cat*, containing the *cat* gene, allowing 2 days for gene expressin. That assay did not detect a difference between BS and normal cell lines. This is probably because pRSV*cat* does not replicate in the human cells and therefore did not need DNA ligase activity for survival. While we were able to detect circular plasmid forms on Southern blots after transfection of linear pZ189, those were not detectable after transfection of pRSV*cat* (data not shown).

After transfection of linear plasmids into BS and normal cells we found a wide variety of mutations at the plasmid joining sites: small and large deletions with or without short sequence homologies, small and large insertions of plasmid sequences or of unidentified (probably cellular) sequences, reverse insertions of plasmid DNA and complex rearrangements. A similar variety of mutations at DNA joining sites were described previously with mammalian cells using SV40 DNA (Woodworth-Gutai, 1981; Wilson *et al.*, 1982; Wake *et al.*, 1984) and plasmid shuttle vector systems (Calos *et al.*, 1983; Razzaque *et al.*, 1984; Hesse *et al.*, 1987; Lieber *et al.*, 1987). Most mutations were deletions and insertions, and in addition, insertions of cellular sequences into the vector (Calos *et al.*, 1983; Razzaque *et al.*, 1984) and filling in of overlapping ends (Wake *et al.*, 1984) were described similar to our findings.

As previously reported in other systems double strand DNA breakage markedly increases the mutation frequency (Razzaque *et al.*, 1984). We found the mutation frequency of linear plasmid to be 250- to 25 000-times higher than the spontaneous mutation frequency with the circular plasmid. Plasmids with overlapping or blunt ends showed a 1.4- to 5.4-fold higher mutation frequency when passed through the BS cells than through normal cells. With the overlapping ended plasmids there was a significant increase in all three categories of mutations (i) deletions at the joining site, (ii) insertions at the joining site or more complex mutations and (iii) point mutations in the *supF* gene) (Table III). With the blunt ended plasmid there was a significant increase in deletions and in point mutations with the fibroblasts (Table II). This is evidence for an error-prone DNA end-joining process in the BS cells. Deletions and, to a great extent, insertions and complex mutations reflect end modifications of the plasmid DNA before joining is completed. A possible explanation for the large increase in mutations is that because of the ligase deficiency the ends of linear plasmids were not joined as rapidly in BS cells as in normal cells and therefore were exposed for a longer time to end modifying processes. An alternative explanation is that an elevated activity of end-modifying enzymes (e.g. exonucleases) or the activity of a defective DNA ligase in BS cells introduced more mutations at the joining sites than in normal cells. Point mutations may reflect defective functioning of the excision repair system which contains a ligase step, or action of an error-prone polymerase entering at a DNA strand break, as proposed previously for pZ189 in xeroderma pigmentosum group A cells (Seidman *et al.*, 1987).

There was an increased spontaneous mutation frequency with the untreated circular plasmid in BS cells in comparison to normal (2.2-times with fibroblasts and 20.8-times with lymphoblasts–Table III). Warren *et al.* (1981) reported a 4- to 15-fold increase in spontaneous 6-thioguanine resistance in BS fibroblasts and Vijayalaxmi *et al.* (1983) reported an 8-fold higher incidence of 6-thioguanine resistance in peripheral blood lymphocytes of BS patients. They found DNA breaks and rearrangements in the hypoxanthine guanine phosphoribosyl transferase gene locus of the patient's lymphocytes chromosomes. Langlois *et al.* (1989) found a 50- to 100-fold increased frequency of NM blood group variant erythrocytes with partial or total loss of expression of gene locus. With our plasmid we found spontaneous insertions and complex mutations only with the BS cells. The frequency of point mutations was increased 1.7- to 11.3-fold with the BS cells in comparison to the normal

cells. In a circular plasmid, insertions or complex mutations require DNA double strand breaks and ligation, processes that are assumed to be involved in SCEs, chromosome breakage and rearrangements, and genetic recombination. Since DNA double strand breaks occur spontaneously in DNA transfected into mammalian cells (Wilson *et al.*, 1982; Wake *et al.*, 1984), DNA ligase deficiency could contribute to increases in insertions and more complex mutations. The SCE rate in the BS cells we studied was 60–80 per cell compared to 4–8 in normal cells (Willis *et al.*, 1987). The elevated rate of insertions or complex mutations in the untreated plasmid passaged through the BS cells might be based on similar processes responsible for the elevated SCE, and chromosome breakage and rearrangements in BS.

Patients with BS have an increased susceptibility to bacterial infections. A number of immune abnormalities have been found in different BS patients including low immunoglobulin levels, reduced reactivity to skin test antigens *in vivo*, low *in vitro* proliferative response to mitogen stimulation with reduced production of all classes of immunoglobulin, depressed natural killer cell activity, impairment of B cell differentiation and reduced helper T cell regulatory function (Hütteroth *et al.*, 1975; Weemaes *et al.*, 1979; Taniguchi *et al.*, 1982; Ueno *et al.*, 1985). Some of these immune defects may be related to abnormal gene processing. Normal immunoglobulin gene processing involves DNA rearrangements with random joining of V, D and J segments (combinatorial joining), variability in the positin of joining (junctional diversity), nucleotide addition in heavy chain genes and hypermutation (Watson *et al.*, 1987). These functions involve DNA joining with deletions or insertions, DNA rearrangements and inversions, and hypermutability, all of which were observed to be increased in our plasmids passaged through the BS lines. Recent studies (Hesse *et al.*, 1987; Lieber *et al.*, 1987) utilizing plasmid vectors containing portions of immunoglobulin germ line genes have demonstrated the utility of the approach of using plasmid vectors in examining the mechanisms of generation of immune diversity. Our studies demonstrate a reduced DNA end-joining ability, an error-prone DNA end-joining, and spontaneous hypermutability of plasmids in BS cells. These defective processes may be involved in the increased chromosome breakage and the impaired immune function of patients with BS.

Materials and methods

Cells

The Simian virus 40 (SV40) transformed fibroblast cell lines GM8505A (BS) and GM0637 (normal), and Epstein–Barr virus transformed lymphoblast cell lines GM3403C (BS) and GM0606 (normal) were obtained from the Human Genetic Mutant Cell Repository, Camden, NJ and grown as described previously (Protić-Sabljić *et al.*, 1985). Doubling times were 37 h for BS fibroblasts, 32 h for normal fibroblasts, 80 h for BS lymphoblasts and 54 h for normal lymphoblasts.

Plasmid and transfection

The replicating shuttle vector plasmid pZ189 (Seidman *et al.*, 1985, 1987; Bredberg *et al.*, 1986) is shown in Figure 1. It was cut with the restriction endonucleases *Eco*RI at bp 1 (4 bp overlapping ends) or *Stu*I at bp 3474 (blunt ends). Complete cleavage (less than 0.05% circular forms remaining) was verified by agarose gel electrophoresis and Southern blotting. Fibroblasts were transfected by the calcium phosphate–DNA precipitation method (Protić-Sabljić *et al.*, 1985), using 5 μg of untreated circular plasmid or 20 μg of linearized plasmid and 2×10^6 cells/100-mm tissue culture dish for each sample. Lymphoblasts were transfected by the DEAE–dextran method (Seidman *et al.*, 1985), using 5 μg of plasmid and 30×10^6 cells/T75 flask for each sample. Two to three days after cell transfection plasmid DNA was extracted by a rapid alkali lysis procedure (Hesse *et al.*, 1987). Digestion with *Dpn*I was used to remove the unreplicated input plasmid DNA (*Dpn*I does not cut DNA with a mammalian methylation pattern) (Seidman *et al.*, 1985).

Bacterial transformation and mutant analysis

Purified plasmid was introduced into competent *Escherichia coli* MBM7070 and spread on to LB agar dishes with ampicillin, supplemented with Xgal and IPTG (Seidman *et al.*, 1985). Many mutations in the *supF* tRNA gene of the plasmid yield white or light blue bacterial colonies, whereas the intact *supF* gene results in blue colonies (Kraemer and Seidman, 1989). The colonies of two or three transformations in each sample were scored. A representative sample of plasmids from blue, light blue and white colonies was isolated from 5-ml overnight cultures (Birnboim and Doly, 1979) and analyzed by agarose gel electrophoresis (to determine the size of the plasmid compared to the wild-type) and digestion with *Eco*RI or *Stu*I (to screen for restitution of the restriction site). The base sequence of the *Eco*RI and *Stu*I restriction site regions and the *supF* gene of 53 plasmids passed through fibroblasts was determined using a dideoxy procedure with a modified T7 polymerase (Sequenase™, United States Biochemicals, OH).

Acknowledgements

We thank Dr R.Tarone for assistance in the statistical analysis of the data. T.M.R. is supported by a grant from the Deutsche Forschungsgemeninschaft through the Christian-Albrecht University Kiel, FRG

References

Arrand,J.E. Willis,A.E., Goldsmith,I. and Lindahl,T. (1986) *J. Biol. Chem.*, **261**, 9879–9882.

Birnboim,H.C. and Doly,J. (1979) *Nucleic Acids Res.*, **7**, 1513–1523.

Bredberg,A., Kraemer,K.H. and Seidman,M.M. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 8273–8277.

Bubley,G.J. and Schnipper,L.E. (1987) *Somatic Cell Mol. Genet.*, **13**, 111–117.

Calos,M.P., Lebkowski,J.S. and Botchan,M.R. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3015–3019.

Chan,J.Y.H., Becker,F.F., German,J. and Ray,J.H. (1987) *Nature*, **3??**, 357–359.

German,J. (1983) In German,J. (ed.), *Chromosome Mutation and Neoplasia.* Alan R.Liss, New York, pp. 97–134.

German,J., Bloom,D. and Passarge,E. (1984) *Clin. Genet.*, **25**, 166–174.

Gretzula,J.C., Hevia,O. and Weber,P.J. (1987) *J. Am. Acad. Dermatol.*, **17**, 479–488.

Hesse,J.E., Lieber,M.R., Gellert,M. and Mizuuchi,K. (1987) *Cell*, **49**, 775–783.

Hütteroth,T.H., Litwin,S.D. and German,J. (1975) *J. Clin. Invest.*, **56**, 1–7.

Kraemer,K.H. and Seidman,M.. (1989) *Mutat. Res.*, in press.

Langlois,R.G., Bigbee,W.L., Jensen,R.H. and German,J. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 670–674.

Lieber,M.R., Hesse,J.E., Mizuuchi,K. and Gellert,M. (1987) *Genes Dev.*, **1**, 751–761.

Protić-Sabljić,M. and Kraemer,K.H. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 6622–6626.

Razzaque,A., Chakrabarti,S., Joffee,S. and Seidman,M.M. (1984) *Mol. Cell. Biol.*, **4**, 435–441.

Rünger,T.M. and Kraemer,K.H. (1988) In Friedberg,E. and Hanawalt,P. (eds), *Mechanisms and Consequences of DNA Damage Processing, UCLA Symposia on Molecular and Cellular Biology.* Alan R.Liss, New York, new series 83, pp. 381–386.

Seetharam,S., Protić-Sabljić,M., Seidman,M.M. and Kraemer,K.H. (1987) *J. Clin. Invest.*, **80**, 1613–1617.

Seidman.M.M., Bredberg,A., Seetharam,S. and Kraemer,K.H. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 4944–4948.

Seidman,M.M., Dixon,K., Razzaque,A., Zagursky,R.J. and Berman,M.L. (1985) *Gene*, **38**, 233–237.

Söderhäll,S. and Lindal,T. (1976) *FEBS Lett.*, **67**, 1–8.

Taniguchi,N., Mukai,M., Nagaoki,T., Miyawaki,T., Moriya,N., Takahashi,H. and Kondo,N. (1982) *Clin. Immunol. Immunopathol.*, **22**, 247–258.

Ueno,Y., Miyawaki,T., Seki,H., Hara,K., Sato,T., Taniguchi,N., Takahashi,H. and Kondo,N. (1985) *Clin. Immunol. Immunopathol.*, **35**, 226–233.

Vijayalaxmi, Evans,H.J., Ray,J.H. and German,J. (1983) *Science*, **221**, 851–853.
Wake,C.T., Gudewicz,T., Porter,T., White,A. and Wilson,J.H. (1984) *Mol. Cell. Biol.*, **4**, 387–398.
Warren,S.T., Schultz,R.A., Chang,C.C., Wade,M.H. and Trosko,J.E. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 3133–3137.
Watson,J.D., Hopkins,N.H., Roberts,J.W., Steitz,J.A. and Weiner,A.M. (1987) *Molecular Biology of the Gene*. Benjamin/Cummings, Menlo Park, CA, Vol. II, pp. 832–894.
Weemaes,C.M.R., Bakkeren,J.A.J., Ter Haar,B.G.A., Hustinx,T.W.J. and van Munster,P.J.J. (1979) *Clin. Immunol. Immunopath.*, **12**, 12–19.
Willis,A.E. and Lindahl,T. (1987) *Nature*, **327**, 355–357.
Willis,A.E., Weksberg,R., Tomlinson,S. and Lindahl,T. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 8016–8020.
Wilson,J.H., Berget,P.B. and Pipas,J.M. (1982) *Mol. Cell. Biol.*, **2**, 1258–1269.
Woodworth-Gutai,M. (1981) *Virology*, **109**, 353–365.

Received on February 17, 1989