Elevated spontaneous mutation rate in Bloom syndrome fibroblasts
(mutator gene/carcinogenesis/somatic mutations)

STEPHEN T. WARREN, ROGER A. SCHULTZ, CHIA-CHENG CHANG, MARGARET H. WADE, AND JAMES E. TROSKO

Department of Pediatrics and Human Development, College of Human Medicine, Michigan State University, East Lansing, Michigan 48824

Communicated by James V. Neel, February 4, 1981

ABSTRACT The rates of spontaneous mutation to 6-thioguanine resistance were determined in fibroblasts derived from normal and two Bloom syndrome individuals (GM 2548 and GM 1492). Two methods were utilized to determine the rates. Method I obtained the spontaneous mutation rate from the increase in the mutation frequency of a cell population in logarithmic-phase growth over 10 days. The two Bloom syndrome strains had spontaneous mutation rates of $16 \times 10^{-6}$ and $17 \times 10^{-6}$ mutations per cell per generation, whereas two normal strains had rates of $1.5 \times 10^{-6}$ and $1.1 \times 10^{-6}$. Method II utilized fluctuation analysis to measure the rate of spontaneous mutation. This method resulted in rates of $19 \times 10^{-6}$ and $23 \times 10^{-6}$ mutations per cell per generation in Bloom syndrome cells, compared to rates of $4.6 \times 10^{-6}$ and $4.9 \times 10^{-6}$ in the control strains. These data suggest that Bloom syndrome may be a mutator mutation, a previously unrecognized phenomenon in humans, and that an elevated spontaneous mutation rate in vivo may be responsible for the clinical phenotype of primordial dwarfism and increased cancer incidence.

Bloom syndrome is a rare, autosomal recessive human disorder phenotypically characterized by primordial dwarfism, facial telangiectasia, immunopathy, and a distinct propensity to develop cancer at a relatively early age (1–5). Cytologically, cells obtained from patients with Bloom syndrome (genotypically designated bl/bl) exhibit increased chromosomal instability, particularly breakage and homologous rearrangements (6–8). In addition to the chromosomal breakage, these cells have a manyfold increase in sister-chromatid exchanges (SCE) over normal cells, such that a markedly elevated frequency of SCE may be considered pathognomonic of Bloom syndrome (9–11).

Because of the chromosome breakage, elevated SCE frequency, and predisposition to develop cancer, Bloom syndrome frequently has been listed as a DNA repair-defective syndrome (12). Although there have been conflicting reports of cellular sensitivity to UV irradiation or ethylmethylsulphonate exposure (13, 14), various measurements of the DNA repair capacities of Bloom syndrome cells after DNA damage strongly suggest that the repair functions, as presently understood, are intact (15–23). However, evidence provided by Hand and German (24, 25) indicates that DNA replication is abnormal in Bloom syndrome. These authors observed a significantly slower rate of DNA chain growth along the replica in fibroblasts and in lymphocytes obtained from individuals with Bloom syndrome. Although this may be dependent on cell density (26), Giannelli et al. (13) provided further support for the contention that semiconservative replication is abnormal in Bloom syndrome fibroblasts, as is evident from the observations of a delayed rate of DNA chain maturation and a cell-cycling disturbance. DNA replication abnormalities have been shown to influence the degree of fidelity of the replicating unit in prokaryotes (27, 28), and in rodent cells mutator activities have been shown to be associated with mutations that affect DNA replication (29, 30). If we assume, albeit on limited data, that the fundamental defect in Bloom syndrome is related to some aspect of DNA replication, then it is conceivable that the bl gene, when homozygous, may have mutator activity. Therefore, we examined the spontaneous mutation rates of Bloom syndrome fibroblasts by using resistance to the purine analog 6-thioguanine (6SGua) as a genetic marker to test this hypothesis. A preliminary report of this work was presented at the 1980 Meeting of the American Society of Human Genetics (31).

MATERIALS AND METHODS

Cell Strains. Skin fibroblast cultures GM 2548 and GM 1492, originally derived from two patients with Bloom syndrome [Bloom syndrome registry designations 71(HaEn) and 44(ABRo), respectively], were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). Three fibroblast strains, NSF-1, NSF-4, and NSF-791, initiated in this laboratory from fore- skins of three healthy male infants, served as controls. SCE determinations were performed on all lines prior to use as described (32) to confirm their genotypes (bl/bl or +/-). All strains had male karyotypes and were used prior to passage 16.

Culture Conditions. Cells were grown in modified Eagle’s minimal essential medium (33) with Earle’s salts, supplemented with 100% increase of all nonessential amino acids, 50% increase of all vitamins and essential amino acids except glutamine, 1 mM sodium pyruvate, and either 15% (vol/vol) fetal calf serum or 10% fetal calf serum and 5% calf serum (GIBCO). The bicarbonate concentration was decreased to 1 g/liter. The medium also was supplemented with 100 units of penicillin G and 100 μg of streptomycin per ml during the experiments. Cells were incubated at 37°C in humid 95% air/5% CO₂. Cells were removed from plastic surfaces by using 0.01% crystalline trypsin and 0.6 mM EDTA (Sigma) in phosphate-buffered saline without calcium and magnesium ions.

Spontaneous Mutation Rate Experiments. Spontaneous mutation rates were determined by two methods with 6SGua resistance as a genetic marker (34). 6-Thioguanine-resistant (6Gua') cells are presumptive mutants of the X chromosome-linked enzyme hypoxanthine/guanine phosphoribosyltransferase (HGPRT; EC 2.4.2.8).

The first protocol. Method I, was a modification (35) of the technique originally used by Newcombe (36) in bacterial cultures to estimate the spontaneous mutation rate from the increase in the spontaneous mutation frequency during a defined growth period of a cell population. Into five 150-cm² plastic dishes,
flasks (Corning Glass Works), approximately 4 \times 10^6 cells per cell strain were inoculated in growth medium; this initial cell population is designated \( N_1 \). Cell attachment was complete for mass culture of these cell strains. From the same initial population, 4.8 \times 10^6 cells per strain were inoculated in 120 plastic dishes (9 cm; Corning) and treated with 65Gua (10 \mu g/ml; Sigma) for the determination of the initial mutation frequencies, \( MF_1 \). Six additional dishes per strain were each inoculated with 200 cells in growth medium for an estimation of the cloning efficiencies of each strain. The medium in the selection dishes was changed every third or fourth day with fresh medium containing 65Gua. The dishes were stained on the 25th day with crystal violet (2 g/100 ml) in 10% (vol/vol) ethanol, and the colonies were scored (colonies were scored if greater than 50 cells were observed microscopically). The cells in the plates were allowed to grow for 10 days, with the medium changed twice during this period. At the end of 10 days, the cells of each strain were rinsed with phosphate-buffered saline, trypsinized, and pooled for the determination of cell number, designated \( N_2 \). The pooled cells from each strain were plated as described for the determination of the second mutation frequencies, \( MF_2 \), \( N_1 \) and \( N_2 \) were determined from hemacytometer counts on the pooled cells without correction for cloning efficiency.

The second protocol, Method II, was a modification of fluctuation analysis (37, 38). For each strain, cells (50 cells per dish for control strains and 100 cells per dish for the Bloom strains, to compensate for the lowered plating efficiency in Bloom syndrome fibroblasts) were inoculated into each of 96 dishes (9 cm) and grown in nongrowth medium for 15 days with a single medium change at day 8. At the end of this growth period, three dishes per strain were stained, and colonies were counted to determine the initial cell number, \( N_0 \), plated into the dishes. Another five dishes were separately rinsed with saline, and the cells were trypsinized and counted to determine the final cell number, \( N_f \). The cells from these dishes were pooled for each strain, recounted, and diluted to inoculate six culture dishes per strain with 200 cells per dish in order to determine the cloning efficiencies. The cells in the remaining dishes were each rinsed once with saline containing 0.6 mM EDTA, treated for 8 min with 1 ml of 0.01% trypsin (without EDTA), dispersed with Pasteur pipettes (dispersion monitored microscopically), and suspended in the original dishes with 10 ml of medium containing 10 \mu g/ml of 65Gua per ml. It was estimated that each dish contained less than 4.1 \times 10^4 cells. The medium was changed 16 hr later with fresh medium containing 65Gua and was changed thereafter every third day for 22 days. At that time the plates were stained and colonies were counted as described in Method I.

Calculations. The number of cell divisions was determined from the formulae \((N_2 - N_1)/\ln 2\) and \((N_1 - N_0)/\ln 2\) for Methods I and II, respectively. Calculations of mutation frequencies and rates were based on the mean number of mutant colonies per dish, \( m \), which was determined in two ways: (i) \( m = \ln 1/Po \), where \( Po \) equals the percentage of plates with zero 65Gua colonies; and (ii) the arithmetic mean, where \( m = \text{total observed} \times 65Gua \text{colonies per total dishes} \).

For Method I the mutation frequencies, \( MF_1 \) and \( MF_2 \), were determined by dividing \( m \) by the number of cells inoculated per dish (\( 4 \times 10^6 \)) multiplied by the cloning efficiency. The spontaneous mutation rate, \( a \), was then determined by:

\[
a = \frac{2(MF_1 - MF_2)}{\ln(N_2/N_1)} \ln 2. \tag{1}
\]

For the fluctuation analysis (Method II), the spontaneous mutation rate was calculated in two ways. The first was derived from equation 4 of Luria and Delbruck (37):

\[
a = \frac{mC}{(N_1 - N_2)/\ln 2}. \tag{2}
\]

in which \( C \) is the number of replicate cultures (disks). The second calculation of \( a \) is based on equation 8 of Luria and Delbruck (37) as formulated by Capizzi and Jameson (39):

\[
a = \frac{(CN_2)}{CN_1}. \tag{3}
\]

Eq. 3 may be solved by first determining \( Cr \), in which \( r \) is the mean number of 65Gua colonies per dish corrected by the cloning efficiency, thus, \( r = m/CE \). Therefore, the numerator of Eq. 3 (\( CaN_2 \)) may be found based on the quantity \( Cr \) in tables provided by Capizzi and Jameson (39). Note that Eq. 3 is thus corrected for the cloning efficiencies of the cell strains, whereas Eq. 2 is not.

RESULTS

Spontaneous Rate of Mutation from Method I. Table 1 summarizes the cell population growth in the five flasks over the 10-day period for each cell strain examined. The two Bloom syndrome strains GM 2548 and GM 1492 started at a population size of \( 4.0 \times 10^6 \) and \( 4.2 \times 10^6 \) cells, respectively, and increased to \( 15.5 \times 10^6 \) and \( 17.7 \times 10^6 \) cells at the end of the growth period, corresponding to \( 16.6 \times 10^6 \) and \( 19.5 \times 10^6 \) cell divisions for the respective populations (calculated as described).

The two normal strains NSF-1 and NSF-4 began at a population size of \( 4.4 \times 10^6 \) and \( 4.0 \times 10^6 \) and ended at \( 86.0 \times 10^6 \) and \( 82.2 \times 10^6 \) cells, respectively. This represented \( 118 \times 10^6 \) and \( 113 \times 10^6 \) cell divisions for the normal strains, considerably more than the Bloom syndrome strains, which are known to have slower growth rates (26). Also it should be noted that, ideally, the protocol of Method I should be carried out beyond the 10-day growth period. However, our experience indicates that Bloom syndrome cells sense early in culture, thus excluding the extension of the experiment. Table 1 also shows a reduced cloning efficiency for Bloom syndrome fibroblasts as compared to normal human fibroblasts, which again is consistent with the observations of earlier investigators (13, 26).

Data for mutation frequency determinations at two different times (one performed on the cell populations at the start of the experiment and the second performed on the populations after 10 days of growth) are in Table 2. The mutants were selected at a cell density of \( 4 \times 10^6 \) cells per 9-cm dish. Under these conditions, metabolic cooperation, which may lower the recovery of 65Gua mutants, should not be an influence (40, 41). NSF-4 had the lowest number of observable 65Gua colonies, 3 out of 120 dishes, whereas GM 1492 had the highest number of 65Gua colonies with 137 out of 119 plates. Some groups had less than the initial 120 dishes because of loss from contamination. The mean number of mutants per dish, \( m \), was calcu-

<table>
<thead>
<tr>
<th>Table 1. Cell population growth parameters for Method I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell strain</td>
</tr>
<tr>
<td>------------</td>
</tr>
<tr>
<td>GM 2548</td>
</tr>
<tr>
<td>GM 1492</td>
</tr>
<tr>
<td>NSF-1</td>
</tr>
<tr>
<td>NSF-4</td>
</tr>
</tbody>
</table>

\( N_1 \), the cell number at the beginning of the growth period, and \( N_2 \), the cell number at the end of the 10 days of growth, were determined from hemacytometer counts uncorrected for cloning efficiencies (CE, and CE').
lated either from the direct mean observed or from the Po number, which assumes the 6SGua' colonies were in a Poisson distribution among the dishes. The observed and calculated m values were all reasonably close to one another for each strain, which indicates that satellite colony formation did not occur to a significant degree.

Mutation frequencies (Table 3) were calculated by using the data in Tables 1 and 2. The mutation frequencies increased for all four strains over the 10-day growth period, with the two Bloom syndrome strains showing the greatest change. Spontaneous mutation rates for the two Bloom syndrome strains were $16 \times 10^{-6}$ and $17 \times 10^{-6}$ mutations per cell per generation in GM 2548 and GM 1492, respectively. Control strains NSF-1 and NSF-4 had spontaneous mutation rates of $1.5 \times 10^{-6}$ and $1.1 \times 10^{-6}$ mutations per cell per generation, respectively. Rates for the control strains are in close agreement with published rates for normal diploid human fibroblasts at the HGPRT locus (38). These data indicate an approximately 10-fold increase in the spontaneous mutation rate in Bloom syndrome fibroblasts as compared to normal fibroblasts at this locus.

Spontaneous Rate of Mutation from Method II. Results of the fluctuation analysis are shown in Table 4. Ten 6SGua' colonies from 75 replicate cultures (dishes) and 20 6SGua' colonies from 68 cultures were observed in control strains NSF-4 and NSF-791 respectively. In contrast to this, 58 6SGua' colonies from 88 cultures and 43 6SGua' colonies from 79 cultures were observed in Bloom syndrome strains GM 1492 and GM 2548, respectively. The distribution of these resistant colonies among the cultures and the increase of mutant colonies among the Bloom syndrome strains is seen in Fig. 1.

Calculation of the mutation rates (Table 4) was first with Eq. 2, using m derived both from the mean colonies per dish and the Po number. These spontaneous mutation rates again show the Bloom syndrome cells with a higher rate over controls. However, Eq. 2 does not correct for cloning efficiencies, which are considerably lower in the Bloom syndrome cultures. When the cloning efficiencies are incorporated into the calculations by using Eq. 3, all the rates increase; however, the Bloom syndrome rates are significantly greater than the control strains. These data again suggest that Bloom syndrome has an elevated spontaneous mutation rate.

**DISCUSSION**

The results of these experiments demonstrate that fibroblasts from patients with Bloom syndrome have a 5- to 10-fold increase in their spontaneous mutation rates at the HGPRT locus over control fibroblasts. Because the rate was shown to be elevated through two separate methods and because other investigators (38, 42) have presented evidence that resistance to 6SGua (at 10 μg/ml) appears to be the result of a genetic event at the locus.
coding for HGPRT (as opposed to a phenocopy or epigenetic event), we believe the gene $bl$, when homozygous, may have significant mutator activity associated with it. Similar supporting data from other loci would be needed to confirm this hypothesis. However, other genetic loci now used as selective systems in human fibroblasts, such as diphtheria toxin resistance (elongation factor 2 locus) or ouabain resistance ($Na^+$,$K^+$-ATPase locus), do not lend themselves readily to spontaneous mutation rate experiments because they are associated with significant selective disadvantages in culture or have relatively low mutation frequencies (43, 44), or both.

It is tempting to correlate the possible mutator activity with the clinical phenotype of Bloom syndrome patients. These individuals have significantly low birth weights and remain small in stature throughout their relatively short lifetime (2, 5). Because the rate of growth of an individual is fundamentally determined by cell number (45), cell loss caused by inviable somatic mutations, in addition to a slow mitotic rate, may play an important role in Bloom syndrome. Patients with Bloom syndrome also have an extremely high incidence of malignancy; therefore, the correlation with a high spontaneous mutation rate and the somatic mutational theory of cancer should be readily apparent (5, 46). The yield of spontaneous mutants is intimately correlated with the number of cell divisions (47, 48); thus, it is interesting that the majority of cancers in Bloom syndrome patients are found in tissues of high proliferation, such as the bone marrow and gastrointestinal tract (49). Furthermore, the observation of German et al. (10) that in some patients with Bloom syndrome a small proportion of lymphocytes exhibit a normal SCE frequency could be explained by a somatic back mutation to $+/bl$ in a clone of progenitor stem cells.

Because Bloom syndrome cells have elevated chromosome breakage and SCE, it is conceivable that mechanisms enhancing either deletions or unequal exchanges in the region distal to Xq26, where HGPRT maps (50), is responsible for the apparent mutator activity. Alternatively, the enhanced mutation rate may be the result of an abnormality in DNA replication. Because DNA polymerase activities appear normal in Bloom syndrome fibroblasts (51), it is possible that replication is aberrant due to influences such as abnormal deoxyribonucleoside triphosphate pools. Meuth et al. (29) and Chang et al. (30) have demonstrated mutator activity associated with Chinese hamster cell mutants having altered DNA precursor pools or slower growth rates. Furthermore, unbalanced deoxyribonucleoside triphosphate pools may lead to elevations in SCE frequencies (52, 53).

In conclusion, these experiments support the hypothesis that Bloom syndrome fibroblasts have an elevated spontaneous mutation rate as compared with control fibroblasts. The in vitro cellular characteristics of slow growth rate and higher spontaneous mutation rate possibly associated with the $bl/bl$ genotype is, in our opinion, consistent with the clinical phenotype. However, until further elucidation of the basic defect in Bloom syndrome is reached, the mechanism of the hypermutability remains unknown.

**Note Added in Proof.** Gupta and Goldstein (54) have reported corroborating data by finding an elevated spontaneous mutation rate to diphtheria toxin resistance in one Bloom syndrome fibroblast strain, GM 1492.

The authors wish to acknowledge Ms. Beth Rupp for her excellent technical assistance and Ms. Judy Copeman for her typing assistance. This research was supported by Grant 1R01 CA 56903 to J.E.T. from the National Cancer Institute and a Young Environmental Scientist Award ES01809 to C.-C.C. from the National Institute of Environmental Health Sciences.

etics 50, 151–156.