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Altered temporal expression of DNA repair in hypermutable Bloom's syndrome cells

(cell proliferation/spontaneous mutagenesis/uracil DNA glycosylase/gene regulation/human genetic disorders)

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ABSTRACT **The temporal regulation of DNA repair during synchronous cell proliferation was examined in normal human skin fibroblasts and in Bloom's syndrome skin fibroblasts. Normal human cells regulated DNA repair in a defined temporal sequence prior to the induction of DNA replication. Nucleotide-excision repair was stimulated prior to the induction of base-excision repair, which itself was increased prior to the induction of DNA replication. This temporal sequence was observed (*i*) by quantitation of the induction of the base-excision repair enzyme uracil DNA glycosylase during cell proliferation in the absence of cellular insult and (*ii*) by quantitation of nucleotide-excision repair after UV irradiation or base-excision repair after exposure to methylmethane sulfonate. In contrast, Bloom's syndrome cells were characterized by specific alterations in this temporal sequence of gene regulation, such that DNA repair was not enhanced prior to the induction of DNA replication. Nucleotide-excision repair, base-excision repair, and the uracil DNA glycosylase were induced in a temporal sequence identical to that observed for DNA polymerase and for DNA replication. The inability of Bloom's syndrome cells to enhance DNA repair prior to DNA replication suggests that miscoding lesions remain in DNA and are replicated during cell proliferation.**

Recent studies have examined the enzymatic mechanisms through which human cells recognize and correct perturbations in DNA structure. Two major excision-repair pathways have been identified: (*i*) nucleotide-excision repair, in which DNA adducts are removed within oligonucleotides, and (*ii*) base-excision repair, in which DNA adducts are removed as modified bases leaving an apurinic or apyrimidinic site in DNA (1–3). Recent results from this laboratory have suggested that human cells regulate these enzymatic pathways during cell proliferation in a defined and ordered temporal sequence with respect to DNA replication (4–8). In particular, nucleotide-excision repair and base-excision repair reached their peak activity several hours prior to DNA replication. The base-excision repair enzyme uracil DNA glycosylase was induced prior to the induction of DNA polymerase during cell proliferation in the absence of environmental insult as a normal modulation of enzyme activity. We have suggested that this temporal sequence of DNA repair capacity may function in human cells as a protective mechanism designed to decrease mutagenesis or carcinogenesis. The prior stimulation of DNA repair would serve as a prescreening mechanism to remove potentially miscoding lesions from DNA to ensure the fidelity of DNA synthesis (9, 10). Alterations in this temporal sequence in human cells such that DNA would not be prescreened prior to replication would be expected to lead to increased rates of mutagenesis or oncogenesis (11, 12).

Recently, Warren *et al.* (13, 14) and Gupta and Goldstein (15) reported that Bloom's syndrome cells could be characterized by abnormally high spontaneous mutation rates. Individuals with Bloom's syndrome have an increased rate of neoplasia (16) and their cells exhibit high incidences of chromosomal aberrations (17, 18). Warren *et al.* (14) suggested that alterations in DNA polymerase producing a mutator polymerase might be responsible for their observations. Using the identical cell strains used by Warren *et al.* (14), we examined whether Bloom's syndrome cells differed in the extent or temporal sequence of DNA repair expression during synchronous cell proliferation. We have found that neither nucleotide-excision repair nor base-excision repair was enhanced prior to the induction of DNA replication. Instead, the expression of each DNA repair pathway as well as the induction of the uracil DNA glycosylase was coordinate with the induction of DNA replication and of DNA polymerase. These results suggest that Bloom's syndrome cells may be characterized by an inability to prescreen DNA prior to replication.

MATERIALS AND METHODS

Cell Culture. Normal human skin fibroblasts (CRL 1222 and CRL 1147) were purchased from the American Type Culture Collection. Bloom's syndrome skin fibroblasts were purchased from the Institute for Medical Research (Camden, NJ) (GM-1492, a 15-yr-old white male, registry no. BS-44, AeRu; GM-2548, a 6-yr-old black male, registry no. BS-71, HaEn). Fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum/12 mM sodium bicarbonate/2 mM glutamine/streptomycin (100 μg/ml)/penicillin (100 units/ml). Fibroblasts were synchronized by maintenance in medium containing 0.3% serum for 5 days and then stimulated to proliferate by the addition of fresh medium containing 20% fetal calf serum. As defined by autoradiographic analysis, <5% of the quiescent cells were synthesizing DNA prior to serum stimulation. Similar analysis showed that >60% of the cells were stimulated to proliferate by serum addition. The absence of mycoplasma contamination was confirmed by the thymidine incorporation test (19).

Enzyme Assays. DNA polymerase was assayed in a total volume of 100 μl of 50 mM Tris·HCl, pH 7.4/5 mM $MgCl_2$/100 μM dATP/100 μM dCTP/100 μM dGTP/100 μM [α-^{32}P]dTTP (500–1,000 cpm/pmol)/7.5 μg of "activated" calf thymus DNA/1 mM dithiothreitol. After incubation for 60 min at 37°C, reactions were terminated by the addition of 1 ml of 10% trichloroacetic acid and 100 μl of heat-denatured calf thymus DNA (1 mg/ml). Acid precipitable radioactivity was determined in a liquid scintillation counter. Uracil DNA glycosylase was assayed in a total volume of 100 μl of 100

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mM Tris·HCl, pH 8.0/10 mM dipotassium EDTA/1 mM dithiothreitol/5 μg of [^{3}H]uracil-labeled calf thymus DNA (750 cpm/pmol)/0.5 μl of cell-free extract. Incubations were carried out at 37°C for 30 min. Reactions were terminated by sequentially adding 60 μl of 2 M NaCl, 300 μl of ethanol, and 100 μl of denatured calf thymus DNA (1 mg/ml). After a minimum of 60 min at −20°C, the mixture was centrifuged at 2,300 × g for 10 min. An aliquot of the ethanol supernatant (200 μl) was assayed to determine the release of [^{3}H]uracil.

DNA Repair. DNA repair was measured as unscheduled DNA synthesis in the presence of freshly prepared 10 mM hydroxyurea or as repair replication into parental DNA in the absence of hydroxyurea. Nucleotide-excision repair was determined as described (6). For quantitation of unscheduled DNA synthesis, unirradiated cultures containing only 10 mM hydroxyurea served as controls. Base-excision repair was assayed by addition of 2 mM methylmethane sulfonate to cells incubated with 10 mM hydroxyurea (5, 6). Control samples were treated indentically except that no methylmethane sulfonate was added. Unscheduled DNA synthesis was determined by the incorporation of [^{3}H]thymidine into acid precipitable material. For repair replication, cells were incubated for 60 min with 5′-bromodeoxyuridine (3 μg/ml) and fluorodeoxyuridine (0.25 μg/ml) and then exposed to either 2 mM methylmethane sulfonate or to UV irradiation (20 J/m^2). Repair replication was determined as described (5, 6).

RESULTS

Regulation of the Uracil DNA Glycosylase During Synchronous Cell Proliferation. Our previous results with WI-38 embryonic lung fibroblasts indicated that the uracil DNA glycosylase was induced during synchronous cell proliferation prior to the induction of DNA polymerase and DNA replication (5–8). As shown in Fig. 1, a similar temporal sequence of enzyme activity could also be observed in normal human skin fibroblasts. At 0 hr, on serum addition, the residual amount of DNA synthesis was 4.1% of that observed when DNA replication was maximally induced. DNA synthesis started to increase 20 hr after serum addition and was maximal after 28 hr (Fig. 1*A*). DNA polymerase was increased 1.9-fold with an induction profile similar to that observed for DNA replication (Fig. 1*B*; 220.33 ± 8.7 pmol per culture vs. 115.88 ± 4.3 pmol per culture at 28 and 0 hr, respectively; $P < 0.05$ by Student's t test). The activity of the uracil DNA glycosylase was examined using the identical cell-free extracts used to quantitate polymerase activity. As shown in Fig. 1*B* the glycosylase was increased 2.5-fold during synchronous cell growth. Glycosylase activity started to increase 6 hr after serum addition and was maximal 20 hr after cell stimulation (337.87 ± 35.40 pmol per culture vs. 137.17 ± 53.84 pmol per culture at 20 and 0 hr, respectively). This increase was significant at $P < 0.05$ as defined by the Student's t test. At maximal glycosylase induction, DNA synthesis was enhanced only to 30% of its maximum level and DNA polymerase reached only 47% of its maximum. After 28 hr, when DNA synthesis and DNA polymerase had increased to their peak rates, uracil DNA glycosylase activity decreased to 30% of its maximal level observed at 18 hr. In this experiment, as defined by [^{3}H]thymidine incorporation into DNA, S phase occurred at later intervals, ≈28 hr after serum addition. Irrespective of this delay in their proliferative response, the temporal regulation of the glycosylase in normal human skin fibroblasts prior to the induction of DNA polymerase and DNA replication was identical to that observed in human embryonic lung cells (5–8). Furthermore, previous studies showed that human cells kept quiescent for equivalent intervals do not change their DNA repair capacity in a qualitative or quantitative manner (5, 6, 8).

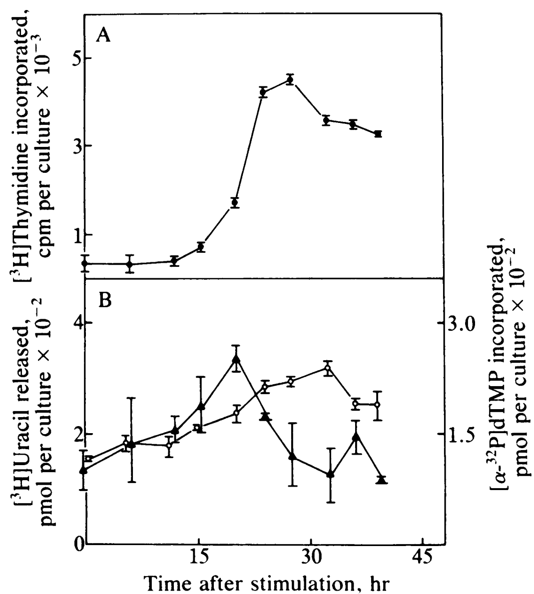

FIG. 1. Regulation of DNA repair in normal human cells during synchronous cell proliferation. Fibroblasts were synchronized by serum depletion and then stimulated to proliferate by the addition of fresh medium containing 20% fetal calf serum. To determine DNA synthesis, cells were pulse-labeled at the indicated intervals with [^{3}H]thymidine (2 μCi per culture, 67 Ci/mmol; 1 Ci = 37 GBq) for 30 min prior to collection. Uracil DNA glycosylase and DNA polymerase activities were determined in *in vitro* reactions. The same cell-free extracts were used to quantitate each enzyme. ▲, Uracil DNA glycosylase activity; ○, DNA polymerase activity; ●, DNA synthesis.

The temporal expression of the uracil DNA glycosylase in Bloom's syndrome cells was then examined. As shown in Fig. 2, Bloom's syndrome cells (GM-1492) were serum-stimulated to traverse two cell cycles. At 0 hr, residual DNA synthesis was 2.8% of that observed when DNA replication was maximally increased. DNA synthesis (Fig. 2*A*) and DNA polymerase (Fig. 2*B*) were maximal 21 hr after cell

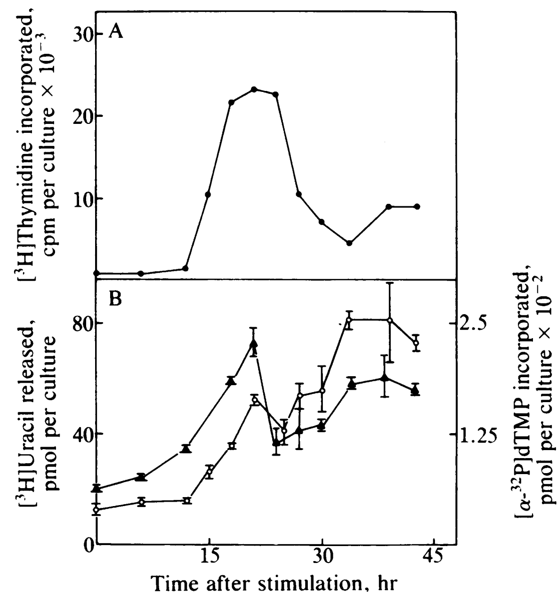

FIG. 2. Regulation of DNA repair in Bloom's syndrome cells during synchronous cell proliferation. Bloom's syndrome cells were cultured and induction of DNA replication, DNA polymerase, and uracil DNA glycosylase were examined. The absolute numbers of each parameter should not be compared with those in normal cells (Fig. 1) as the experiments were not done simultaneously and the number of cells per culture was not identical. ▲, Uracil DNA glycosylase activity; ○, DNA polymerase activity; ●, DNA synthesis.

Table 1. Regulation of DNA repair in normal skin fibroblasts

Time after stimulation, hr	DNA repair: Nucleotide-excision repair, cpm per culture	DNA repair: Nucleotide-excision repair, % of maximal induction	DNA repair: Base-excision repair, cpm per culture	DNA repair: Base-excision repair, % of maximal induction	DNA replication: [^{3}H]Thymidine incorporation, cpm per culture	DNA replication: % of maximal induction
0	828 ± 343	17.9	290 ± 106	5.4	620 ± 28	6.3
6	1,078 ± 272	23.3	450 ± 99	8.4	595 ± 35	6.1
12	1,450 ± 262	31.3	965 ± 233	17.9	585 ± 49	5.9
15	1,375 ± 502	29.7	1,098 ± 237	20.4	1,700 ± 141	17.4
20	2,880 ± 1,216	62.3	2,805 ± 771	52.2	6,690 ± 127	68.4
24	4,625 ± 990	100	2,037 ± 641	37.9	—	—
27	4,313 ± 1,128	93.3	5,378 ± 1,439	100	8,379 ± 530	85.7
29	3,325 ± 1,061	71.9	3,400 ± 559	63.2	9,775 ± 559	100

DNA replication, nucleotide-excision repair after UV irradiation (15 J/m^2), and base-excision repair after 2 mM methylmethane sulfonate exposure were measured. Unscheduled DNA synthesis is expressed as the difference in [^{3}H]thymidine incorporation in the presence of the DNA damaging agent and 10 mM hydroxyurea and that incorporation observed at each interval in cultures pulsed with 10 mM hydroxyurea but not exposed to any DNA damaging agent. As determined by unscheduled DNA synthesis, this temporal sequence of DNA repair activity was observed in normal human skin cells in three separate experiments done at different times and with different lots of serum. Although the absolute intervals at which each activity was maximally increased varied in different experiments, the relative temporal sequence with which nucleotide-excision repair was enhanced prior to base-excision repair, which was enhanced before DNA replication, was invariant within each experiment.

stimulation, and both activities coordinately decreased and were restimulated at later intervals. The 3.9-fold increase in induction of DNA polymerase after 21 hr (163.2 ± 6.8 pmol per culture vs. 41.7 ± 8.1 pmol per culture at 0 hr) is comparable to the extent of induction and the temporal sequence observed in normal human skin fibroblasts and in WI-38 cells. In Bloom's syndrome cells, the uracil DNA glycosylase was induced during synchronous cell growth (73.18 ± 6.9 pmol per culture vs. 20.1 pmol per culture at 21 and 0 hr, respectively). This difference is significant at $P < 0.05$ as defined by Student's t test. This 3.6-fold increase in glycosylase activity in Bloom's syndrome cells was equivalent to the 2.9-fold increase in glycosylase activity observed in the proliferating NHSF cell population. However, the glycosylase was not induced prior to DNA replication or to DNA polymerase. Instead, each activity started to increase 15 hr after stimulation, each was maximally increased at 21 hr, and each declined and was restimulated as the cells traversed a second cell cycle. Previous results showed that normal human cells that were serum-stimulated to traverse two cell cycles induced glycosylase prior to DNA replication or prior to DNA polymerase when each enzymatic activity was examined in the same cell population (5, 8). In contrast, as shown in Fig. 2, in an identical situation, Bloom's syndrome cells failed to enhance the glycosylase prior to DNA replication or prior to the induction of DNA polymerase.

Regulation of DNA Repair During Synchronous Cell Growth. To examine whether alterations in the regulation of the uracil DNA glycosylase in Bloom's syndrome cells might reflect a general alteration in repair regulation, the capacity for nucleotide-excision repair and base-excision repair was compared in normal skin fibroblasts and in Bloom's syndrome cells during synchronous cell growth. As shown in Table 1, nucleotide-excision repair after UV irradiation at 20 J/m^2 was maximally increased 5.3-fold in cultures irradiated 24 hr after cell stimulation (4,625 ± 990 cpm per culture at 24 hr vs. 828 ± 343 cpm per culture at 0 hr). Base-excision repair after methylmethane sulfonate exposure (2 mM) was maximally enhanced 18.5-fold in cultures exposed to methylmethane sulfonate 27 hr after cell stimulation (5,378 ± 1,439 cpm per culture at 27 hr vs. 290 ± 106 cpm per culture at 0 hr). DNA replication reached its peak rate at 29 hr. At this interval, nucleotide-excision repair and base-excision repair had decreased to 71.9% and 63.2%, respectively, of their maximal levels. This temporal sequence of repair activity prior to DNA synthesis as determined by unscheduled DNA synthesis was observed in three separate experiments in normal human skin fibroblasts and is identical to that previously observed in five separate experiments in WI-38 embryonic lung fibroblasts (refs. 5, 6, and 8; unpublished observations).

The temporal expression of DNA repair in Bloom's syndrome cells (GM-1492) was then examined. A typical experi-

Table 2. Regulation of DNA repair in Bloom's syndrome fibroblasts

Time after stimulation, hr	DNA repair: Nucleotide-excision repair, cpm per culture	DNA repair: Nucleotide-excision repair, % of maximal induction	DNA repair: Base-excision repair, cpm per culture	DNA repair: Base-excision repair, % of maximal induction	DNA replication: [^{3}H]Thymidine incorporation, cpm per culture	DNA replication: % of maximal induction
0	3,552 ± 854	16.3	1,618 ± 400	17.4	1,632 ± 16	24.4
6	3,774 ± 1,414	17.4	1,080 ± 594	11.6	754 ± 58	11.2
14	11,130 ± 3,394	51.2	4,407 ± 1,426	47.2	2,985 ± 360	44.7
16	9,875 ± 7,618	45.4	6,503 ± 3,203	69.8	4,443 ± 201	66.5
18	15,835 ± 2,632	72.8	8,397 ± 2,625	90.1	5,404 ± 545	80.1
20	21,747 ± 366	100	9,319 ± 2,954	100	6,683 ± 53	100
22	12,331 ± 1,177	56.7	7,223 ± 1,654	77.5	3,439 ± 303	51.5

The regulation of DNA replication and DNA repair were examined. The absolute hours at which each activity was maximally induced in Bloom's syndrome cells and in normal human skin cells (Table 1) should not be compared as the experiments were not done simultaneously. As determined by unscheduled DNA synthesis, the coordinate expression of DNA repair and DNA replication in Bloom's syndrome cells was observed in two separate experiments. The apparent high basal levels of DNA synthesis at 0 hr may result from the use of [^{3}H]thymidine (67 Ci/mmol) to quantitate residual levels of DNA synthesis prior to serum stimulation.

Table 3. Cell-cycle regulation of DNA repair

		DNA repair				DNA replication	
		Nucleotide-excision repair		Base-excision repair			
Cell source	Time after stimulation, hr	cpm per μg of DNA	% of maximal induction	cpm per μg of DNA	% of maximal induction	[³H]Thymidine incorporation, cpm per culture	% of maximal induction
Normal skin fibroblasts	0	316	50.0	225	34.2	486	12.1
	17	420	66.6	164	24.9	1,505	37.4
	21	631	100	289	43.9	3,279	81.6
	26	293	46.4	658	100	4,019	100
	32	310	49.1	276	41.9	3,790	94.3
Bloom's syndrome	0	482	50.4	146	49.9	595	4.1
	18	445	46.6	221	74.1	2,522	17.5
	24	455	47.6	230	77.8	3,774	26.1
	32	955	100	298	100	14,440	100
	36	642	67.2	198	66.2	11,820	81.9

The induction of DNA replication and the regulation of DNA repair were examined. DNA was isolated according to the procedures of Goth-Goldstein (20). Alkaline cesium chloride equilibrium density analysis was done as described by Pettijohn and Hanawalt (21). DNA that sedimented at the density characteristic of unreplicated DNA was collected. The specific activity of parental DNA was then determined. The concentration of parental DNA was quantitated by absorbance spectroscopy.

ment is shown in Table 2. Nucleotide-excision repair after UV irradiation was increased 5.8-fold in cultures irradiated 20 hr after serum addition (21,747 ± 366 cpm per culture at 20 hr vs. 3,552 ± 854 cpm per culture at 0 hr). This 5.8-fold increase in unscheduled DNA synthesis after UV irradiation was virtually identical to the 6.1-fold increase observed in the proliferating NHSF cell population. Similarly, base-excision repair was enhanced 6-fold in cultures exposed to 2 mM methylmethane sulfonate 20 hr after serum addition (9,319 ± 2,954 cpm per culture at 20 hr vs. 1,618 ± 400 cpm per culture at 0 hr; $P < 0.05$ by Student's t test). At this interval, DNA synthesis was also stimulated to 100% of its maximum. Furthermore, each activity was coordinately decreased at later intervals. Thus, in Bloom's syndrome cells, there was an alteration in the unique temporal sequences with which each repair pathway was enhanced with respect to the induction of DNA replication during synchronous cell proliferation.

To verify temporal perturbations in DNA repair expression in Bloom's syndrome cells as detected by unscheduled DNA synthesis, repair replication into parental DNA during cell proliferation was examined. As shown in Table 3, in normal human skin fibroblasts, nucleotide-excision repair after UV irradiation was increased 2-fold in cultures examined 21 hr after serum stimulation and was decreased at later intervals; base-excision repair was increased 2.5-fold in cultures exposed to 2 mM methylmethane sulfonate at 26 hr and was decreased to basal levels thereafter. DNA replication was maximal 26–32 hr after cell stimulation. This temporal expression of DNA repair in normal human skin cells as determined by repair replication is identical to that previously documented in three separate experiments in WI-38 cells (refs. 5 and 6; unpublished observations). In contrast, as measured by repair replication in Bloom's syndrome cells (GM-2548), nucleotide-excision repair after UV irradiation was increased 2-fold in cultures irradiated 32 hr after serum addition. Base-excision repair was increased 2-fold in cultures exposed to 2 mM methylmethane sulfonate at 32 hr. At this interval, DNA synthesis reached its maximum stimulation. Furthermore, each activity coordinately declined and was decreased at 36 hr.

DISCUSSION

The regulation of gene expression during cell proliferation results in a defined temporal order in the activation of specific genes (22, 23). Discrete cellular functions are induced and repressed at defined intervals in the cell cycle. Thus, although the extent of excision repair capacity can be examined in asynchronously growing homogeneous or heterogeneous cell populations (24–33), synchronous cell populations must be used to examine the temporal relationships of repair regulation as quiescent cells are stimulated to proliferate. In this report, we provide evidence that (*i*) normal human skin fibroblasts regulate DNA repair similarly to that modulation observed in embryonic lung cells and (*ii*) Bloom's syndrome cells may be characterized by three separate and independent temporal alterations in this sequential pattern of repair activity. In particular, temporal alterations in glycosylase induction in Bloom's syndrome cells were observed in the identical cells used to quantitate the regulation of DNA polymerase. These results were observed during cell proliferation in the absence of any physical or chemical insult. Therefore, this temporal aberration in enzyme induction in Bloom's syndrome cells could be attributed only to an intrinsic genetic defect in their pattern of excision repair gene regulation and not to an effect of exogenous agents (34).

Our results also show that individual human cell strains stimulated to proliferate with different serum samples in separate experiments differ with respect to the absolute hourly intervals at which each DNA repair pathway or DNA replication was maximally enhanced. In particular, the absolute hourly intervals at which DNA replication was maximal after serum stimulation varied in 12 separate experiments from 21 to 26 hr in WI-38 embryonic lung fibroblasts and from 26 to 32 hr in normal human skin fibroblasts. There was a similar variability in the absolute hours at which each repair parameter examined was maximally enhanced. Such variability is in contrast to the uniformity expected of a cloned cell line. Thus, one might argue that this inherent variability would preclude an examination of the temporal relationship between the regulation of DNA repair and the induction of DNA replication using diverse human cell strains. However, within each of the 12 separate experiments, the DNA repair parameter examined was enhanced prior to the induction of DNA replication and decreased when DNA synthesis was maximally enhanced. Furthermore, this temporal sequence of enhanced repair capacity prior to DNA synthesis was invariant whether normal human cells were stimulated to traverse one or more cell cycles after release from quiescence. There was a similar variability in the maximal enhancement of DNA replication in two Bloom's syndrome cell strains. In five separate experiments, the absolute hourly intervals at which DNA replication was maximal after serum stimulation

varied from 20 to 22 hr (GM-1492) to 32 hr (GM-2548). However, within each of the five separate experiments, the temporal regulation of each DNA repair capacity examined was coordinate with the induction of DNA replication. This altered temporal regulation of DNA repair was invariant whether Bloom's syndrome cells traversed one or more cell cycles after serum stimulation.

Although environmental exposure may be the primary cause of DNA modification, spontaneous DNA lesions may occur at a physiologically significant rate (35–37). In particular, depurination, depyrimidination, and cytosine deamination produce potentially miscoding lesions in DNA. Apurinic sites in DNA increase the infidelity of DNA synthesis (38, 39); cytosine deamination to uracil is by definition a mutagenic event (40). The persistence of these lesions during S phase would be expected to increase the rate of spontaneous mutagenesis. The inability of Bloom's syndrome cells to enhance DNA repair prior to DNA replication indicates that their capacity to prescreen DNA would solely reside in their constitutive levels of DNA repair present in resting cells. Normal human cells, which increase their repair capacity 3- to 10-fold above basal levels prior to S phase, would be expected to have a lower spontaneous mutation frequency than Bloom's syndrome cells. Thus, Bloom's syndrome cells have a 5- to 10-fold increase in the rate of spontaneous mutagenesis as compared to normal human cells (13–15). Furthermore, they stimulated DNA repair concomitant with DNA replication. This suggests that damaged bases might be repaired after they have been replicated. DNA repair pathways provide for the resynthesis of the correct genetic information through the use of undamaged nucleotide sequences in the complementary strand as template. Thus, DNA polymerases that function in repair synthesis would interpret misincorporated bases in daughter DNA as the correct genetic information, incorporate the stereochemically correct complementary base into parental DNA, and thus fix the mutation (40).

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