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A Manyfold Increase in Sister Chromatid Exchanges in Bloom's Syndrome Lymphocytes

(BrdU substitution/chromosome rearrangement/Fanconi's anemia/ataxia telangiectasia)

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ABSTRACT **Dividing cells from persons with Bloom's syndrome, an autosomal recessive disorder of growth, exhibit increased numbers of chromatid breaks and rearrangements. A highly characteristic feature of the chromosome instability in this syndrome is the tendency for exchanges to occur between *chromatids of homologous chromosomes* at homologous sites. In the present experiments, a cytogenetic technique by which the sister chromatids of a metaphase chromosome are stained differentially has been used to demonstrate a striking and possibly specific, but hitherto unrecognized, increase in the frequency with which *sister chromatids* also exchange segments. The cells were grown in bromodeoxyuridine and stained with 33258 Hoechst and Giemsa. Whereas phytohemagglutinin-stimulated lymphocytes from normal controls had a mean of 6.9 sister chromatid exchanges per metaphase (range 1–14), those from persons with Bloom's syndrome had a mean of 89.0 (range 45–162). Normal frequencies of sister chromatid exchanges were found in cells heterozygous for the Bloom's syndrome gene, and also in cells either homozygous or heterozygous for the genes of the Louis-Bar (ataxia telangiectasia) syndrome and Fanconi's anemia, two other rare disorders characterized by chromosome instability.**

In a differentially stained chromatid interchange configuration discovered during the study, it was possible to determine the new distribution of both sister and nonsister-but-homologous chromatids that had resulted from numerous exchanges. By following shifts in the pattern of staining from chromatid to chromatid, visual evidence was obtained that the quadriradial configurations long recognized as characteristic of Bloom's syndrome represent exchanges between homologous chromosomes, apparently at homologous points.

We postulate that the increase in the frequency of exchanges between nonsister-but-homologous chromatids and those between sister chromatids in Bloom's syndrome represents aspects of one and the same disturbance. A study of this phenomenon in relation to the clinical features of Bloom's syndrome may be helpful eventually in understanding the biological significance of chromatid exchange in somatic cells.

Bloom's syndrome is a rare genetic disorder of man characterized clinically by growth retardation, a sun-sensitive eruption of the face, a disturbance of immune function, and a predisposition to cancer (1, 2). In addition, cultured blood lymphocytes and dermal fibroblasts from affected homozygotes (the genotype of which may be described as *bl/bl*) exhibit an excessive instability of their chromosomes, that is, an increase in the number of chromosome breaks and rearrangements in comparison to that in cells of the genotypes *bl/+* and +/+. The chromosome aberrations observed include chromatid and ioschromatid gaps and breaks, sister-chromatid reunions, polycentric chromosomes, and chromatid interchange configurations (1, 3). The most characteristic aberration to have been observed up until now is a certain type of quadriradial configuration, interpreted as the result of a chromatid interchange between the two homologous chromosomes of a pair, with the points of exchange being at apparently homologous sites, often at or near the centromeres. Although various chromosome pairs may be affected in the interchanges, certain ones are affected preferentially (4).

It has been well established in experiments by others that treatment of normal cells with ultraviolet irradiation and chemical mutagens increases the frequency with which sister chromatid exchange (SCE) occurs (5). Until now, the effect on SCE-induction of the *bl/bl* genotype, which, as has just been mentioned, is known to increase exchanges between homologous but nonsister chromatids, has remained unexplored. This in part has been because the standard technique by which SCEs are demonstrated, tritium autoradiography, is not only difficult but possibly inadequate to yield quantitative data for human chromosomes. Recently, relatively simple techniques became available to demonstrate SCEs in somatic metaphase chromosomes. Latt (6) has observed that the fluorochrome 33258 Hoechst [2-[2-(4-hydroxyphenyl)-6-benzimidazolyl]-6-(1-methyl-4-piperazyl)-benzimidazole·3 HCl] shows greater efficiency of fluorescence when bound to poly(dA-dT) than when bound to poly(dA-BrdU). On the basis of this finding he used 33258 Hoechst fluorescence to report BrdU incorporation into the DNA of phytohemagglutinin-stimulated human lymphocytes. If lymphocytes are allowed to replicate twice in the presence of BrdU and the chromosomes at the second post-replication mitosis are stained with 33258 Hoechst, the chromatid in which both DNA strands contain BrdU fluoresces less intensely in ultraviolet light than does the chromatid in which only one strand is BrdU-substituted. In cells so treated, sites of SCEs are readily recognized by following the bright and dim fluorescent patterns along the lengths of the chromatids. Perry and Wolff (7) have shown that similar differentiation can be observed in hamster cells without resorting to fluorescence microscopy, by prestaining the cells in the 33258 Hoechst and subsequently in Giemsa after appropriate pretreatments. We have made use of these new techniques to compare the SCE frequencies in *bl/bl*, *bl/+*, and +/+ lymphocytes and have, besides confirming the observations of Latt and of Perry and Wolff, discovered that the frequency in *bl/bl* lymphocytes is many times greater than in lymphocytes of the two other genetic constitutions. In addition, we have examined cells from persons with two other of the rare and recessively transmitted disorders in which chromosome in-

Abbreviation: SCE, sister chromatid exchange.

stability is present, the Louis–Bar syndrome (or ataxia-telangiectasia) and Fanconi's anemia (8), and have found them to have SCE frequencies similar to that of the normal controls.

MATERIALS AND METHODS

Materials. Venous blood was drawn into sterile heparinized syringes from the following persons:

7 normal persons (genotype presumptively +/+ at the Bloom's syndrome locus);
3 Bloom's syndrome affected homozygotes (genotype *bl/bl*);
2 unaffected carriers of the Bloom's syndrome gene (genotype *bl/+*);
1 Louis–Bar syndrome affected homozygote;
2 unaffected carriers of the Louis–Bar gene;
2 Fanconi's anemia affected homozygotes;
1 unaffected carrier of the Fanconi's anemia gene.

Cell Culture, Harvest, and Slide Preparation. Lymphocytes from whole heparinized blood were cultured for 72 hr in phytohemagglutinin-containing Eagle's minimal essential medium supplemented with 2.4 mM L-glutamine and 10% fetal calf serum. The medium also contained bromodeoxyuridine, fluorodeoxyuridine, and uridine in concentrations respectively of 0.09 mM, 4 μM, and 6 μM.

Metaphases were accumulated by the addition of Colcemid (Ciba) 1 hr before harvest, at a final concentration of 0.6 mM. Following a 10-min hypotonic treatment in 75 mM KCl, the cells were fixed in a 3:1 methanol:acetic acid mixture, initially for a minute as a pellet and then suspended in freshly added fixative. After two changes of the fixative, suspended cells were dropped onto a dry slide cleaned in 70% ethanol. The fixative was allowed to dry under the warmth of a 70-W electric lamp; as the drop of fixative on the slide began to contract, refraction rings appeared on its receding edge, and at that point gentle blowing was used to speed drying.

Staining and Photography. In the initial experiments, cells on the slides were stained for 12 min in 33258 Hoechst (Farbernwerke Hoechst, Germany) (1.0 μg/ml of demineralized water) and then rinsed and mounted in demineralized water under coverslips sealed with rubber cement. They were studied by fluorescence microscopy using a Zeiss photomicroscope with a fluorescent attachment (BG 12 exciter, 50 barrier, and fl 450 dichroic reflector combination). With this method, differential staining of sister chromatids, and exchanges if present, could be seen readily. However, the fading of chromosome fluorescence after about 1 min proved a serious hindrance in photography, so that in subsequent experiments the following technique for staining was employed. The cells on the slides were stained in the 33258 Hoechst and mounted as described above. The following day the coverslips were removed; the slides were incubated (60°) for 2 hr in 2 × SSC (0.3 M sodium chloride and 0.03 M sodium citrate), and the cells were then stained for 30 min in Giemsa (Harleco-Wright's Giemsa) (5% in pH 6.8 buffer prepared using Gurr's buffer tablets). The slides were allowed to dry, passed through xylol, and mounted in Permount. The preparations were examined using regular or phase-contrast optics. Cells exhibiting differential staining of sister chromatids were photographed using Kodak Panatomic X film and a Zeiss interference filter at a setting of 540 nm. In an effort to avoid bias in selecting cells for study, each of those encountered during the scanning of the slide in lengthwise sweeps was photographed if it appeared intact and free of overlap, until 20 to 25 cells were found. From the photographic prints so obtained, only cells with 46 chromosomes were used for SCE counting in the case of the *bl/+* and +/+ genotypes. In the case of the *bl/bl*, all available cells were retained, because we have learned that breaks and rearrangements in such cells are often responsible for a chromosome number other than 46.

SCEs were sought in 8 × 10 inch enlargements of photographs of from 1 to 25 cells (see Table 1) from each of the individuals in whose cultures metaphases were present. The chromosomes in each cell were identified specifically, and the location of each SCE was recorded. Centromeric exchanges were not recorded because it was impossible to distinguish between a true exchange and a twisting of the chromatid

TABLE 1. *Sister chromatid exchanges in lymphocytes of the various genotypes investigated*

Source of lymphocytes	No. of metaphases photographed	Sister chromatid exchanges: Total observed	Mean per cell	Range per cell
Controls				
St Sch ♂	23	150	6.5	2–11
Cri Mc ♀	23	162	7.0	4–11
Ela Lo ♀	15	106	7.1	1–14
Al Mc ♀	2	20	10.0	9 and 11
Ja Jo ♂	1	2		
Ja Ge ♂	0			
Ra Ch ♂	0			
Combined	64	440	6.9	1–14
Bloom's syndrome homozygotes*				
32(Mi Ko) ♂	25	2406	96.2	60–162
47(Ar Smi) ♂	3	215	71.7	58–89
51(Ke Mc) ♂	6	404	67.3	45–99
Combined	34	3025	89.0	45–162
Bloom's syndrome heterozygotes				
Abb Ko ♀	24	151	6.3	2–14
Ber Ko ♂	25	252	10.5	4–19
Combined	49	403	8.2	2–19
Louis-Bar syndrome homozygote				
Dan Bro ♀	3	10	3.3	2–5
Louis-Bar syndrome heterozygotes				
Nan Bro ♀	9	59	6.6	3–13
Hen Bro ♂	4	40	10.0	8–13
Fanconi's anemia homozygotes				
Mar And ♀	1†	5	5.0	
Cha Due ♂	0			
Fanconi's anemia heterozygote				
Her Tis ♀	5	69	13.8	6–16

* Identified by number and initials as in ref. 1.

† Two additional metaphases were examined by fluorescence microscopy but not photographed. See *Results* section of paper.

which would resemble an exchange. The numbers and locations of exchanges in the photographs were determined independently by two observers. The chromosomes in cells of the *bl/bl* genotype had such high numbers of SCEs that disagreement between the counts of the two observers existed for some cells (but always less than 10% of the total in such cells), and in these an average of the two counts was entered in the record.

RESULTS AND COMMENTS

Cultures of lymphocytes from a total of 18 individuals were prepared. The yield of metaphases in these cultures was in general greatly reduced compared to that in untreated phytohemagglutinin-containing cultures. (During the time when these experiments were being made, routine untreated cultures from a variety of individuals, including many of those used in this experiment, were yielding large numbers of metaphases.) A minority of metaphases present showed differential staining. This is apparent from Table 1, where the number of cells in the second column, if less than twenty, represents all that could be located on several slides. We have no explanation at present for the poor and variable yield of metaphases associated with introduction of the three chemicals into the culture medium.

A comparison of the number of SCEs in cells from the different groups of individuals studied (Table 1) brings out the paper's main point, viz., that SCE frequency is increased more than 12-fold in Bloom's syndrome (Figs. 1, 2). In *bl/bl* cells, SCEs per cell varied from 45 to 162 (mean 89.0), while in all other cells they ranged between 1 and 19. The mean in normal controls was 6.9. Table 1 also demonstrates the absence of an increase of SCEs in cells heterozygous for the gene *bl*. Nor does an increase exist in cells either heterozygous or homozygous for the genes responsible for two other well-studied recessively transmitted disorders associated with chromosome instability. Unfortunately, in the case of the Fanconi's anemia affected homozygotes, only a single Giemsa-stained cell in which SCEs could be counted was available; however, we had studied by fluorescence microscopy, but without photography, two additional cells differentially stained with 33258 Hoechst alone. Even though exact counts of SCEs in these two cells were not made, we recorded that the number was not increased above that in normal cells being studied simultaneously. The consistency of the pattern of SCE from cell to cell in all cultures studied so far permits us to place considerable weight on the observation of a normal pattern in such a small number of cells, and to conclude that no increase in SCE occurs in Fanconi's anemia lymphocytes.

We performed a statistical analysis of the SCE data from all photographed cells from three normal controls (St Sch, Cri Mc, and Ela Lo in Table 1), the two carriers of the Bloom's syndrome gene, and one Bloom's syndrome affected homozygote [32(Mi Ko) in Table 1]. The data were first analyzed to learn how the breakpoints were distributed in the genome. The observed distribution of breakpoints producing the SCEs was compared to a distribution calculated on the assumption that the breaks in the different chromosomes are proportional in their frequency to the total relative length of the different chromosomes in the genome. These data are presented in Table 2. χ^2 tests showed a significant deviation between the observed and expected distributions ($P < 0.001$ for all three groups). Inspection of Table 2 reveals that fewer than expected breaks occurred in the chromosomes of groups E, F, and G. A basis for this might lie in our rejection of exchanges in the region of the centromere, a region which constitutes a relatively larger proportion of the length of these chromosomes compared to the longer chromosomes in the genome. Latt (11) observed a similar lower-than-expected SCE frequency in the shorter chromosomes.

DISCUSSION

In their classic experiment on the replication of *Vicia faba* chromosomes using tritium autoradiography, Taylor *et al.* (12) demonstrated that chromosome DNA replication is semiconservative. When chromosomes are labeled during the DNA synthetic phase of one cell cycle and then allowed to

TABLE 2. *The distribution of observed and expected breakpoints that gave rise to sister chromatid exchanges in cells of genotypes +/+, bl/+, and bl/bl*

Chromosome, number or group	Total relative length*	Number of breakpoints†								
		+/+			*bl/+*			*bl/bl*		
		Obs	Exp	χ^2	Obs	Exp	χ^2	Obs	Exp	χ^2
A-1	18.16	35	36.41	0.05	34	35.10	0.03	233	209.57	2.61
A-2	16.90	36	34.12	0.10	48	32.64	7.27	221	196.34	3.11
A-3	14.12	44	28.30	8.71	30	27.28	0.27	197	163.89	7.14
B(4–5)	25.36	67	50.84	5.16	72	48.76	11.06	338	292.58	7.04
C(6–12,X)	78.06	169	156.64	0.98	181	150.72	6.09	923	900.95	0.54
D(13–15)	21.10	41	42.32	0.04	47	40.70	1.46	257	243.50	0.75
E(16–18)	18.28	20	36.62	7.52	8	35.46	21.33	149	210.77	18.21
F(19–20)	8.50	3	17.01	11.52	1	16.52	14.54	38	97.92	36.64
G(21–22,Y)	8.00	3	16.05	10.69	2	16.52	12.73	50	92.39	19.46
Total	208.48	418	418.31	44.77‡	403	403.70	74.78‡	2406	2406.98	95.41‡

* Based on chromosome measurements provided in ref. 9, and calculated as in ref. 10. One and one half times the length of the X chromosome was added to the total relative length of group C and one and one half the length of the Y chromosome was added to the total length of group G.

† Cells used in the analysis are: 23 each from St Sch and Cri Mc, and 15 from Ela Lo of +/+ genotype; 24 from Abb Ko and 25 from Ber Ko of *bl/+* genotype, and 25 from Mi Ko of *bl/bl* genotype (see Table 1).

‡ $P < 0.001$.

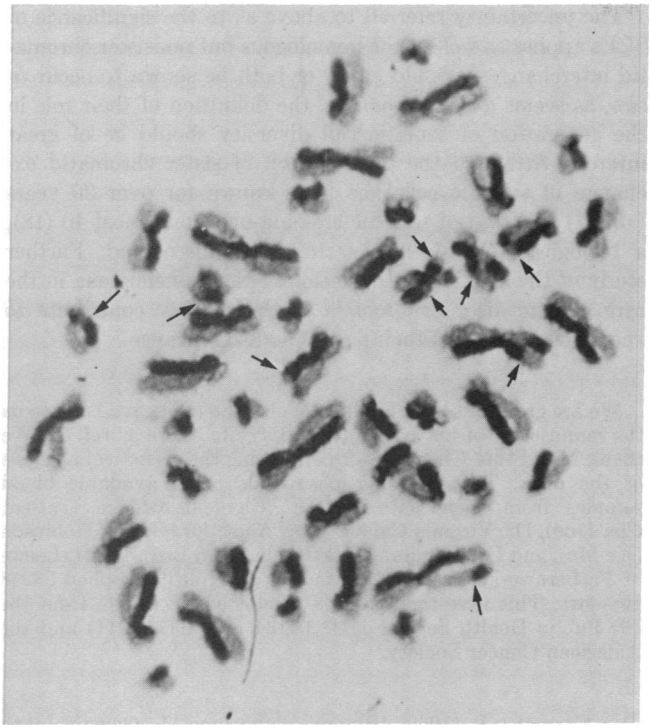


FIG. 1. Chromosomes of a normal lymphocyte at the second metaphase after growth in bromodeoxyuridine, fluorodeoxyuridine, and uridine, stained first with 33258 Hoechst and a day later with Giemsa. Arrows are at points of exchange between sister chromatids (SCE). Enlarged 1800×.

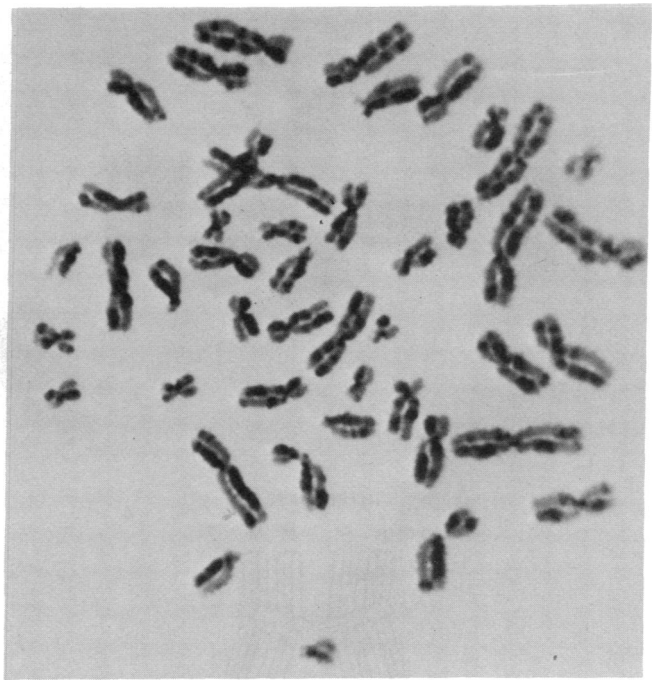
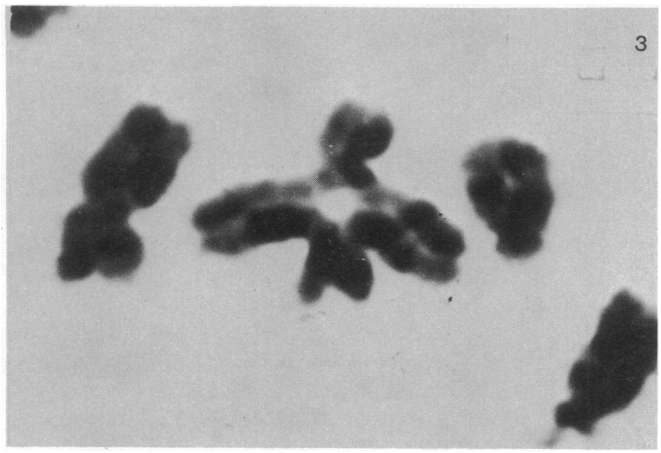


FIG. 2. Chromosomes of a Bloom's syndrome lymphocyte cultured and stained as in Fig. 1, showing many more SCEs than normal (compare with Fig. 1). Enlarged 1800×.

undergo an additional replication in the absence of the label, only one of each pair of daughter chromatids at the second metaphase shows the label. In experiments of similar design studying the chromosomes of *Bellevalia romana*, Taylor (13) observed exchanges between the two sister chromatids of the second mitosis following the labeling. Even though his observation of SCEs has been confirmed in cells of a number of different species, it is still uncertain as to whether these exchanges are spontaneous or radiation induced (14). The demonstration of SCEs in cells from experiments employing no radioactive isotopes indicates that they are not solely radiation-induced artefacts. Also, Latt (6) has found that their frequency did not differ in experiments using isotopically labeled and unlabeled BrdU. The possibility that BrdU might itself induce SCEs must be considered, however. Our present findings lend support to the view that they are not just experimental artefacts. Thus, in cells homozygous for a specific abnormal gene, the number of SCEs was found to be increased, and these are cells which display increased numbers of chromatid breaks and rearrangements when they grow in non-BrdU-containing medium; furthermore, the breaks and rearrangements are of the type that occurs spontaneously in cultured but otherwise untreated cells from normal persons, but at a low frequency.

Our study leaves no doubt that cells homozygous for the Bloom's syndrome gene exhibit a striking increase in the number of SCEs (Table 1, Figs. 1 and 2). Whereas normal cells develop but 1 to 14 exchanges, *bl/bl* cells develop so many that accurate enumeration sometimes becomes difficult. The degree to which *bl/bl* cells differ from the normal is so striking and consistent that observation of just one or two cells is sufficient to determine whether the characteristic pattern is present or absent; the ranges in numbers of SCEs for *bl/bl* and +/+ cells have not been found to overlap. In addition, the *bl/bl* pattern appears so far to be specific. The *bl/+* cell resembles the +/+ in number of SCEs (Table 1). In cells from homozygotes and heterozygotes of the Louis-Bar syndrome and Fanconi's anemia, two other rare autosomal recessive syndromes in which chromosome instability and a predisposition to cancer figure prominently, the frequency of SCEs appears to be the same as that in the normal (Table 1).

Our observations suggest strongly that one and the same disturbance in Bloom's syndrome cells is responsible for the increased exchange between sister chromatids and that between homologous but nonsister chromatids. If so, does it involve a breakage and reunion mechanism akin perhaps to that in effect in meiosis? Several models put forward to explain meiotic exchanges (15) can also be considered in relation to mitotic homologous chromatid interchange of the type seen in Bloom's syndrome (16). The interchange configuration illustrated in Figs. 3 and 4 is significant in this connection. It is one of the three found in the *bl/bl* cells during the present study. To produce the pattern of BrdU incorporation observed in the various chromatids of the two probably homologous chromosomes, one exchange must have taken place between nonsister chromatids at or near the centromeres, as well as a series of exchanges between sister chromatids (see legends of the figures). This observation constitutes the first visual evidence that the quadriradial configuration of Bloom's syndrome actually is the result of exchanges between homologous, but nonsister, chromatids at apparently homologous sites. (It follows that this same interpretation, with the same genetic implications, applies also to similar interchange figures which are, as mentioned earlier, to be found occasionally in cultured cells from normal persons.)



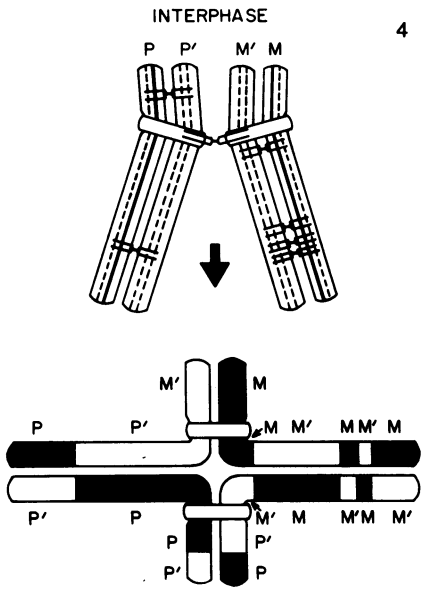


FIG. 3 (*top*). Chromatid interchange configuration composed of two probably homologous chromosomes, from a Bloom's syndrome lymphocyte cultured and stained as in Fig. 1. An interpretation of the complicated staining pattern is presented in Fig. 4. Enlarged 4150×.

FIG. 4 (*bottom*). Diagrammatic representation of chromatid exchanges, both intra- and interchromosomal, to explain the staining pattern in the two homologous chromosomes which compose the quadriradial configuration shown in Fig. 3. *Above*, the two homologous chromosomes are referred to as PP′ (for paternally derived) and MM′ (maternally derived). One of the sister chromatids of each chromosome (sister P and sister M) bears one DNA strand with its normal thymidine content (unbroken line) and one in which thymidine has been replaced by BrdU (broken line). Both strands of the other two sisters (P′ and M′) have only BrdU-substituted strands. Six points of exchange between sister chromatids (SCEs) and one between nonsister chromatids are represented (⊃⊂). *Below*, the composition of chromatids, now different from above as result of the various exchanges, can be determined by following the alternation of the dark and light staining segments (and by our designation of the origin of segments as P, P′, M, or M′). Note that two of the derived chromatids consist now solely of material from the parent chromosome (chromatid P′ PPP′ and chromatid MMM′ MM′M). The other two chromatids have, as result of the exchange between nonsister chromatids, achieved genetic diversity by being composed of segments of both parent chromosomes (chromatid M′P′P and chromatid PP′M′MM′MM′).

The uncertainty referred to above as to the significance of SCEs applies as well to the homologous but nonsister chromatid interchanges. Should either or both be shown to occur *in vivo*, as seems entirely possible, the definition of their role in the generation of somatic cell diversity should be of great interest. Although the phenomenon of sister chromatid exchange in somatic cells has been known for over 20 years (13, 17) and that of somatic crossing-over for almost 40 (18), a biological role for neither has been discovered. Further study of the striking accentuation of these phenomena in the rare genetic disorder Bloom's syndrome may contribute to the understanding of their nature and significance.

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