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Genetics

Molecular evidence that homologous recombination occurs in proliferating human somatic cells

(Bloom's syndrome/loss of heterozygosity/somatic crossing-over/somatic recombination)

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ABSTRACT **A strategy has been developed to detect and characterize certain heritable genomic alterations that occur as cells proliferate *in vitro*. Multiple subclones of cells were isolated from two clonal lymphoblastoid cell lines—one from a boy with Bloom's syndrome (BS), a cancer-predisposing condition known to feature excessive somatic mutation, the other from a normal man. The DNAs from the cell lines were hybridized to a panel of probes that can detect restriction fragment length polymorphisms, and the patterns of polymorphism in the primary clones were compared with that in each of the secondary clones. In one of the BS secondary clones three loci, positioned distally on the long arm of chromosome 3 and that are heterozygous in the donor and all other cell lines derived from the primary clone, had lost heterozygosity and apparently had become homozygous; in contrast, heterozygous loci more proximal on 3q had retained their heterozygosity, as had those on 3p. Taking into account the pattern of chromosome instability uniquely characteristic of BS, the most plausible explanation for the alterations in the altered clone is that somatic recombination had occurred *in vitro*, via homologous chromatid interchange. Such spontaneous recombinational events in nonneoplastic, nonmutagenized cells may contribute to the high cancer incidence in BS and, by analogy, to cancer that arises in the general population.**

Approximately 50 years ago Stern used genetic evidence to demonstrate that somatic recombination is not limited to cells of the germ line in *Drosophila* (1). Then, approximately 25 years ago cytological evidence that crossing-over can occur in mammalian somatic cells was reported (2). The cytological evidence consisted of certain symmetrical four-armed chromosome configurations (Qrs) that occasionally are found in mitotic cells from cultures of human cells (Fig. 1). Such Qrs were interpreted to be the consequence of a segmental interchange between the two chromosomes of a pair, with points of exchange at apparently homologous sites (2). A decade later, application of the then newly available Latt technique for differential staining of sister chromatids provided further cytological support for this interpretation (3).

These observations, on the one hand in dipteran and on the other in human material, had identified crossing-over as a cellular mechanism by which single somatic cells and their progeny might become genetically different from the other cells of the host, specifically by developing homozygosity for an entire segment of a chromosome pair. The observations suggested "the feasibility of detecting a recombination of genes *in vitro* in mammalian cell systems" (2). This now has been accomplished, for both rodent and human cells (4–6). The present experiment also succeeds in detecting somatic recombination, the "genes" examined here, however, being polymorphic segments of DNA rather than coding sequences with recognizable phenotypic effects.

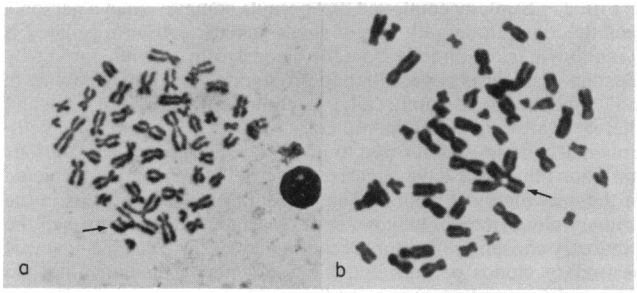

FIG. 1. Metaphases from two blood lymphocytes that had been stimulated to divide *in vitro* by phytohemagglutinin [reproduced from ref. 2 with permission (copyright American Association for the Advancement of Science)]. The four-armed configuration (Qr) (arrows) in each metaphase is the microscopically detectable consequence of an interchange that had occurred at the cell's preceding S phase. The points of exchange in each Qr were at apparently homologous sites near the centromeres in a sister chromatid of each no. 1 chromosome.

The strategy employed to detect "a recombination of genes" was (*i*) to choose at random and isolate a small number of human cells from a large clonal population that had been proliferating *in vitro* for many generations and then (*ii*) to compare the composition of the DNA at selected marker loci in each isolated cell with that of the parental population, searching specifically for loss *in vitro* of constitutional heterozygosity and a corresponding acquisition of homozygosity.

MATERIALS AND METHODS

Cell Lines. A lymphoblastoid cell line (LCL) was developed from each of two healthy individuals. By the technique employed (7, 8), each line was a clone, derived from a single B lymphocyte that had been isolated from freshly drawn blood and transformed by Epstein–Barr virus. One of the clonal LCLs was developed from a boy with Bloom's syndrome (BS) (9), an individual identified as 81(MaGrou) in the Bloom's Syndrome Registry (10). This *primary clone*, named HG 1525, retains the elevated frequency of sister-chromatid exchange (SCE) uniquely characteristic of BS cells (3, 11) and has a Qr incidence of 3 per 1000 metaphases. The same technique was employed to develop a second LCL, HG 1522, but using a lymphocyte from a normal man. Cells from primary clone HG 1522 have, as expected, a normal (low) SCE rate; Qrs have not been observed in it (unpublished data).

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Abbreviations: BS, Bloom's syndrome; LCL, lymphoblastoid cell line; Qr, quadriradial chromosome configuration; SCE, sister-chromatid exchange.

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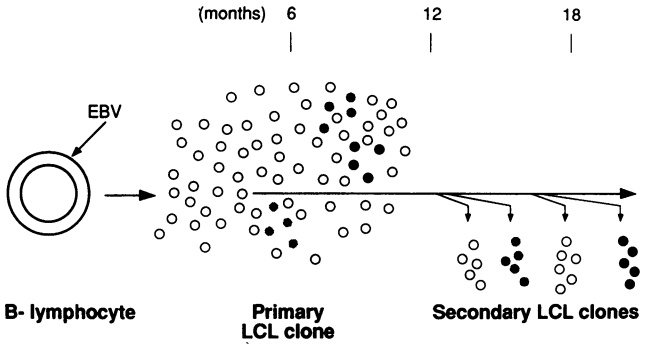

FIG. 2. Strategy employed that permits comparison of a person's genetic constitution with that of single somatic cells. An isolated B lymphocyte taken directly from the bloodstream and transformed by Epstein–Barr virus is permitted to proliferate in culture, doubling in cell number approximately daily. After more than a year *in vitro*, and therefore after several hundred cell divisions, single cells are withdrawn at random and allowed to proliferate into subclones. Various mutations that may have occurred prior to the subcloning (depicted in the drawing by symbols different from those of most cells in the primary clone) may or may not be represented in the genomes of the randomly chosen progenitors of the subclones. The establishment of secondary clones permits detection of genomic changes that had occurred *in vitro* because the background of genotypes of cells that either did not change or had changed in different ways is ablated. In the present experiments, mutations were sought in clones derived from a boy with BS, cell line HG 1525, and from a normal man, cell line HG 1522. EBV, Epstein–Barr virus.

Subcloning of Cell Lines. Both cell lines were allowed to proliferate freely for more than a year at 37°C in medium RPMI 1640 (GIBCO) containing 20% heat-inactivated (57°C, 30 min) fetal bovine serum, penicillin (100 international units/ml), streptomycin (100 μg/ml), and 2.4 mM L-glutamine. Both cultures have a population doubling time of approximately a day, so that several hundred cell-division cycles had occurred *in vitro*. (The experimental design is depicted in Fig. 2.) After the year had passed, single cells were isolated from these primary clones and permitted to give rise to *secondary clones*: on the 385th day of culture of primary clone HG 1525, and again on the 415th day, a random sample of cells was withdrawn and transferred to 96-well plates at a concentration of one cell per well. Each well also contained 10^4 feeder cells (x-irradiated 46,XX lymphoblastoid cells). After 4–6 weeks of incubation, wells that contained actively proliferating cells were identified and the cells from each were expanded into the secondary clones. Similarly, multiple subclones were developed from primary clone HG 1522 on days 362, 388, and 418.

Restriction Fragment Length Polymorphism Analysis. DNA probes for a number of polymorphic loci throughout the genome that were heterozygous in the primary clones with respect to restriction fragment length—the probes and loci will be identified below—were hybridized to appropriately digested DNA from the secondary clones. In this experiment, the DNAs from 67 secondary clones were examined; these in effect were the DNAs from 67 randomly chosen cells, half from the BS primary clone and half from the normal. High molecular weight DNA was isolated from the cell lines by a conventional method (12). Restriction enzymes and random primer labeling kits were obtained from Boehringer Mannheim, and reactions were carried out according to the manufacturer's directions. Southern blots were made using Biotrans nylon membrane from ICN according to the manufacturer's directions. Hybridizations were performed at 65°C in 50 mM sodium phosphate (pH 7.5), 10 mM EDTA, 5% dextran sulfate salt, 4× SSC (1× SSC = 0.15 M NaCl/15 mM sodium citrate), 0.2% SDS, 1× Denhardt's solution (0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone), and 125 μg of salmon testis DNA per ml. Final washes were performed at 65°C in 0.1× SSC/0.1% SDS.

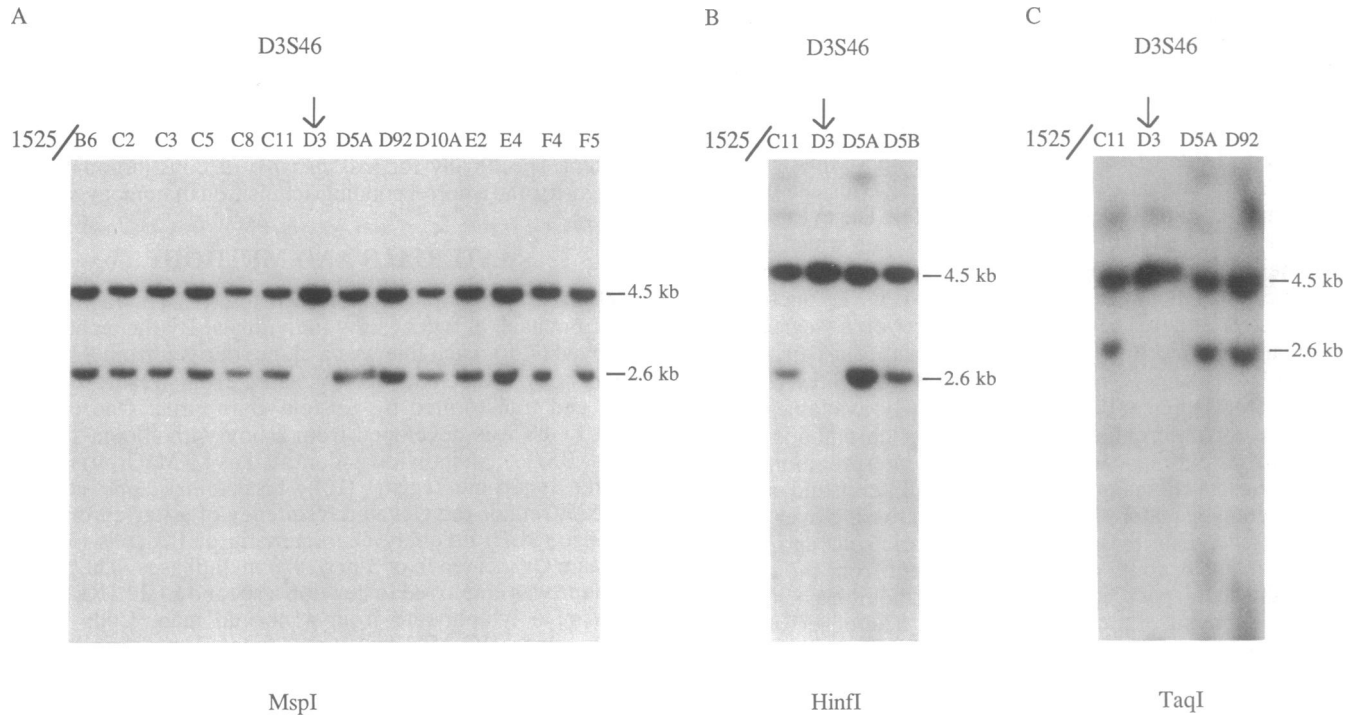

FIG. 3. Molecular evidence for acquisition of homozygosity at locus *D3S46*, a locus identified by the probe and digestions indicated in Fig. 4. DNA was extracted from 34 secondary clones derived from HG 1525, digested with various restriction enzymes, and probed with pEFD64.2. (*A*) Southern blot analysis of 14 of the 34 secondary clones digested with *Msp* I. In all except clone 1525/D3 (arrow), the progeny of a cell removed from HG 1525 on the 415th day of its *in vitro* life, a 4.5-kilobase (kb) and a 2.6-kb fragment were identified, the same as in the child's fibroblasts in long-term culture (not shown). (*B* and *C*) Blots from four secondary clones from HG 1525 as in *A* but digested with enzymes *Hin*fI (*B*) and *Taq* I (*C*).

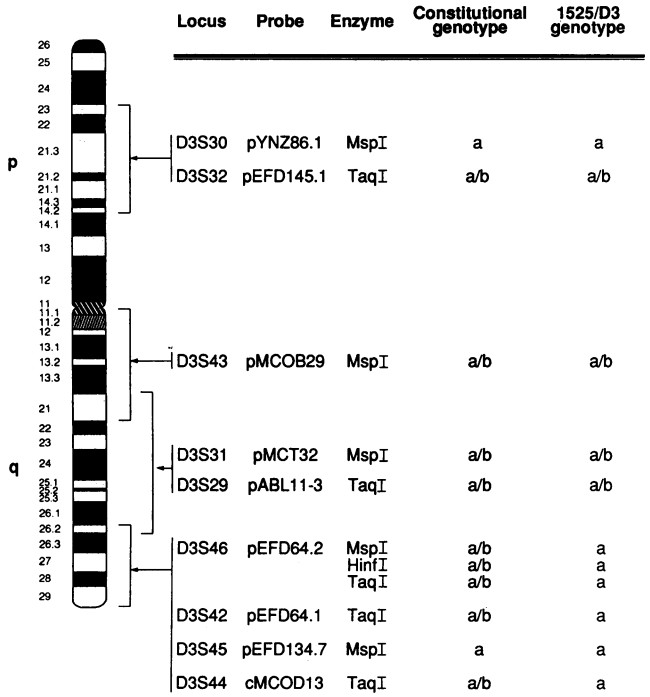

FIG. 4. Polymorphic loci on chromosome 3 examined for possible *in vitro* mutation, indicating their approximate positions along the chromosome, the probes and restriction enzymes used to identify them (refs. 14–21; Y.N. and R. White, unpublished data), and their zygosity in the individual with BS from whom primary clone HG 1525 was derived (column 4) and in secondary clone 1525/D3 (column 5). [Notes: (*i*) Letters a and b refer to the alleles detected at a given locus, with no implications with respect to hemi- vs. homozygosity when a single allele was detected. (*ii*) *D3S43* is not mapped further than to mid- or proximal 3q. (*iii*) Relative positions of the four loci in the linkage group on distal 3q have not been determined.]

RESULTS

The search for acquisition *in vitro* of homozygosity in the secondary clones included the examination of, among others, a VNTR (variable number of tandem repeat) marker (13), pEFD64.2, which identifies locus *D3S46* near the distal end of the long arm (q) of chromosome 3. (VNTR loci are highly polymorphic, so that heterozygosity at such a locus is likely to exist in any one individual, as proved to be the case at *D3S46* in both of the donors of the cell lines examined here.) When pEFD64.2 was hybridized to *Msp* I-digested DNA from primary clones HG 1525 and HG 1522, the same two polymorphic restriction fragments—alleles—were recognized in each. The present report concerns this locus primarily, along with certain others on chromosome 3 that became relevant once change at *D3S46* was detected in one of the secondary clones.

In 66 of the 67 secondary clones examined, no alteration at *D3S46* had occurred, the two parental alleles appearing in every lane of the Southern blots. *Msp* I-digested DNA of several of the secondary clones from HG 1525 are shown in Fig. 3*A*, all but one of which have both a 4.5-kb and a 2.6-kb allele, as did cultured fibroblasts from the same BS child (not shown here). However, as shown in Fig. 3*A* (arrow), pEFD64.2 did detect an alteration in *Msp* I-digested DNA from one secondary clone, clone 1525/D3. In contrast to all others from HG 1525 and all from HG 1522, secondary clone 1525/D3 lacked the 2.6-kb allele of the parental DNA and, in addition, exhibited an increase in intensity of the 4.5-kb allele. (Hybridization of this blot with a control probe, for a locus not mapping to chromosome 3, showed that all lanes were loaded with DNA approximately equally. Also, ethidium bromide staining of the gel showed no significant quantitative differences between lanes.)

DNA from 1525/D3 was digested with two additional restriction enzymes that reveal the same polymorphism as *Msp* I—namely, *Hin*fI and *Taq* I. The same loss of heterozygosity was demonstrated, again with loss of the 2.6-kb allele (Fig. 3 *B* and *C*, arrows).

Further analysis of the two no. 3 chromosomes of secondary clone 1525/D3 then was made by examining other potentially informative polymorphic loci on 3q and 3p (Fig. 4). Four loci were shown to have retained their heterozygosity [*D3S32*, *D3S43*, *D3S31*, and *D3S29* (Fig. 5)] and two to have lost heterozygosity [*D3S44* and *D3S42* (Fig. 6)].

Finally, cytogenetic examination of 20 metaphases from 1525/D3 showed that each cell had two normally G-banded no. 3 chromosomes.

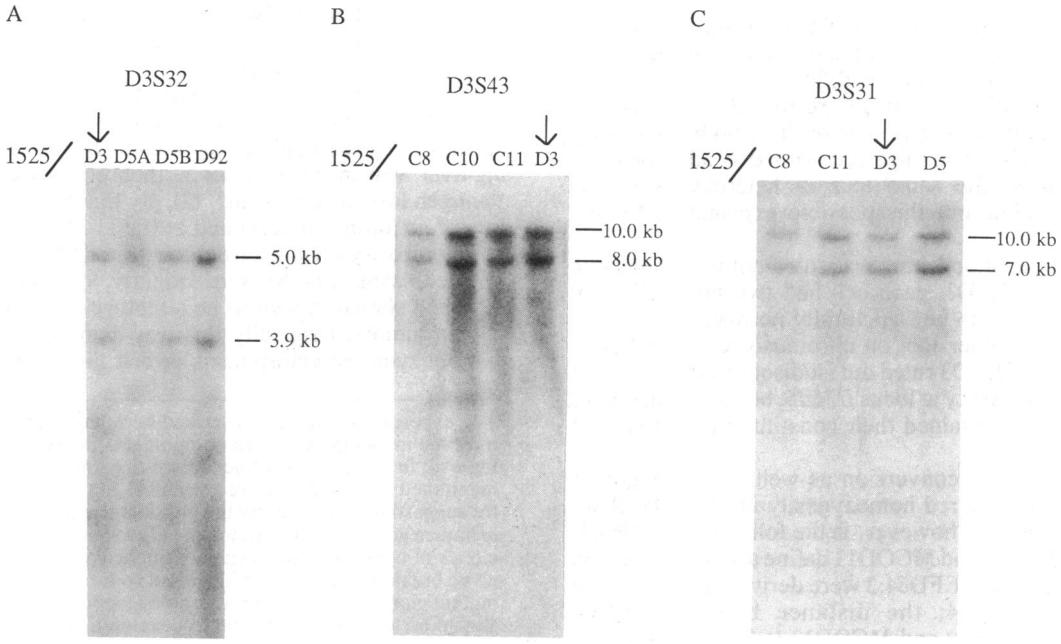

FIG. 5. Molecular evidence for retention of heterozygosity in DNA from clone 1525/D3 at informative loci *D3S32*, *D3S43*, and *D3S31*. These loci are identified by the probes and enzymes indicated in Fig. 4. Heterozygosity was retained also at *D3S29* (not shown here).

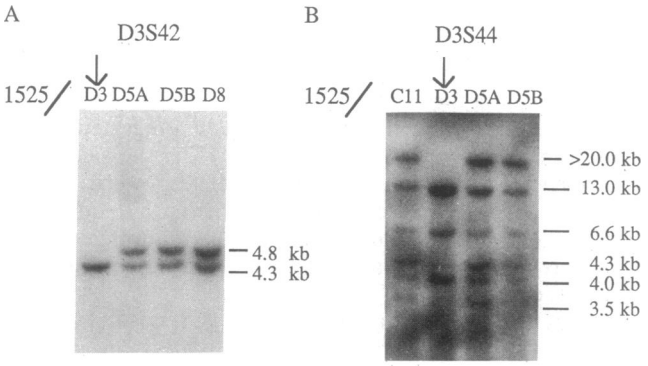

FIG. 6. Molecular evidence for loss of heterozygosity in DNA from clone 1525/D3 at informative loci *D3S44* and *D3S42*, loci identified by the probes and enzymes indicated in Fig. 4.

DISCUSSION

The Qrs that constituted the original evidence that somatic crossing-over occurs in human cells (2, 3, 11, 22, 23) are detected much more readily in cells from persons with the rare recessively transmitted disorder BS (9) than in cells from either normal persons or persons with any other known genetic disorder. Also, genetically marked viruses placed in BS cells experimentally undergo recombination more frequently than when placed in normal cells (24, 25). In theory, the study of BS cells enhances the chance of obtaining experimental evidence of somatic recombination. Accordingly, one of the two primary clones employed here was derived from an individual with BS. This cell line, HG 1525, presents the characteristic and distinctive cytological phenotype of BS: an elevated SCE rate (3, 11) and Qrs in ≈0.3% of metaphases. The other cell line, HG 1522, was derived from a healthy normal man and, as expected, had few SCEs per cell. Of the 67 secondary clones examined in the present experiments for evidence of somatic mutations (including recombination), 33 were from the person with BS and 34 were from the normal person.

The discovery in secondary clone 1525/D3 not only of loss of the 2.6-kb band at locus *D3S46* but also of an increase in intensity of the remaining (4.5 kb) band is interpreted to indicate acquisition *in vitro* of homozygosity of the locus by the clone and a corresponding loss of heterozygosity. Several mechanisms by which this alteration in phenotype might have come about were considered, as follow.

(*i*) Base substitution within one of the *Msp* I restriction sites could explain the altered size of the 2.6-kb allele. But, when 1525/D3 DNA was digested with two additional restriction enzymes, the same loss of heterozygosity was demonstrated, ruling out this possible explanation for the observation.

(*ii*) Chromosome monosomy was ruled out by cytogenetic examination of 1525/D3; each cell had two no. 3 chromosomes that appeared to be structurally normal.

(*iii*) Analysis of other loci on chromosome 3 in DNA of secondary clone 1525/D3 ruled out isodisomy as the cause of the shift to homozygosity at locus *D3S46*, because other more proximal loci on 3q retained their constitutional patterns of heterozygosity.

(*iv*) In theory, gene conversion as well as crossing-over could explain the acquired homozygosity in 1525/D3. Relevant to this possibility, however, is the following: EFD64.1, EFD64.2, EFD134.7, and MCOD13 define a linkage group on distal 3q; EFD64.1 and EFD64.2 were derived from the same cosmid clone, EFD64; the distance between EFD64 (EFD64.1 and EFD64.2) and MCOD13 is 5–7 centimorgans (Y.N. and R. White, unpublished data). It seems improbable that conversion could affect such a large tract of DNA.

(*v*) The alterations detected at loci *D3S46*, *D3S44*, and *D3S42* are the findings expected if crossing-over had occurred in 3q in an ancestor of the progenitor cell of clone 1525/D3. The cytogenetics of BS, specifically the evidence that Qrs are so readily detectable in lymphoid cells from persons with BS, militates strongly for somatic crossing-over as the explanation for the changes detected. Fig. 4 shows that the postulated point of homologous exchange is distal to *D3S29* and proximal to the linkage group of which locus *D3S46* is a member. Thus, we conclude that the mutational event responsible for our results is somatic crossing-over (26).

The frequency of crossing-over cannot be estimated from the present experiments, nor is it known when during the life of HG 1525 the mutational event took place. The formation of a Qr is a rare event even in BS cells. If the development of homozygosity of a given chromosome segment should provide a selective proliferative advantage or disadvantage to the affected cell and its progeny, the ease or difficulty of its detection would be affected, further obfuscating the frequency of somatic recombination. The only safe conclusion is that recombinational events can and do take place but that they are rare in cell lines proliferating *in vitro*, including BS lines. [So far in our survey of mutation in BS cells, examples of loss of heterozygosity have been detected in at least 11 chromosome arms other than 3q (27). Further work is necessary to determine whether these other losses of heterozygosity also are the result of recombination.]

It was explained above that BS cells were employed experimentally here only to enhance the chance of success in the detection of somatic cell recombinational events.¶ However, because all of the cytogenetic changes that characterize BS cells are to be found also in cells from normal persons (though in much lower frequency), it is expected that molecular evidence for crossing-over can be found also in HG 1522, the line derived from the normal person, if more loci are probed and if additional secondary clones from the line are examined. BS is an experimental model. Because the cytogenetic abnormalities of BS cells are quantitatively rather than qualitatively different from cells of normal persons, the findings in BS apply in principle to normal cells. Furthermore, even though the genomic instability of BS was first demonstrated *in vitro* (2, 3, 11, 22, 23, 28, 29), it now is clear that it occurs *in vivo* as well (30–33). Therefore, data of the type obtained in the present experiments that employed cultured BS cells presumably apply as well to cells proliferating *in vivo* in normal persons.

With respect to cancer, the demonstration that a segment of a chromosome can become homozygous in an untreated somatic cell is particularly significant. In 1964 somatic crossing-over was mentioned as a theoretical explanation for "antigen loss in neoplasms" (2). By that time the occasional loss of an antigen determined at the H-2 locus—i.e., loss of heterozygosity—had been demonstrated in experimental murine neoplasms (34–36). Subsequently, loss of heterozygosity has been shown as well to be a feature of standard types of human tumors. Originally this was demonstrated by examining enzyme polymorphisms in cell lines derived from tu-

¶The experimental system described here has applicability in chromosome mapping. Although potential usefulness of somatic crossing-over in human cells for demonstrating linkage relationships was mentioned in 1964 (2), we are grateful to John H. Edwards (Oxford) for suggesting to us recently the possible usefulness of our cell lines in human gene mapping. Although only a small number of secondary clones of cells with homozygosity of chromosome arms distal to a given breakpoint have been identified so far, should the use of such mutant clones in human chromosome mapping become desirable, it should be technically possible to develop a panel of cell lines with points-of-exchange widely distributed over the various chromosome arms. Such "recombined" lines would reveal the relative positions of loci already assigned to specific arms by other methods.

mors (36, 37) but more recently by examining DNA polymorphisms in tumors themselves (38–45). In many cases the basis for the loss(es) of heterozygosity is not demonstrable, but somatic recombination, along with other cytological mechanisms, does appear to be important in the neoplastic process, possibly in multiple ways and at multiple stages of initiation and progression.

Regarding initiation of neoplasia, once a somatic cell or cell population becomes encumbered with a recessive mutation that affects one allele of certain loci concerned with proliferative control, crossing-over is available as a chromosome mechanism by which that mutation can be advanced to homozygosity (46), thereby providing some degree of growth autonomy for that cell and its progeny. The original mutation can have been inherited or it can have occurred *de novo* (47, 48). In this view, the original heterozygous mutant cell and its progeny would be considered a *preneoplastic population*, the homozygous population *neoplastic*. That crossing-over actually can result in neoplasia was first demonstrated in the rare tumor retinoblastoma (38).

In relation to these earliest events that provide growth autonomy to a cell and the lineage to which it gives rise, the significance of the present work is that loss of heterozygosity has been demonstrated in *nonneoplastic* cells taken from a healthy person—notably, however, from a person who is homozygous for the BS mutation. BS more than any other known human disease, and more than any cancer-promoting environmental situation, predisposes affected individuals to cancer of the sites and types that affect the general population (49, 50). As reported elsewhere, BS cells do accumulate an abnormally greater number of mutations (28–33, 51), and the mutations are of various types; many involve entire segments of chromosomes, and a proportion of them are best explained by somatic recombination (32). This is evidence that nonneoplastic tissues composed of cells in which recombination and other mutations occur with increased frequency constitute an unusually dangerous milieu from which neoplasia probably will emerge, and further, that among those mutations are represented the type(s) that are essential first events in neoplastic transformation, not just consequences of it. By this interpretation, one or more of the mechanisms that generate somatic mutations in nonneoplastic BS cells—including crossing-over—represent the types of mechanisms that generate mutations in normal people, although less frequently than in BS.

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