Proc. Nat. Acad. Sci. USA
Vol. 71, No. 8 pp. 3162–3166 August 1974

Sister Chromatid Exchanges, Indices of Human Chromosome Damage and Repair: Detection by Fluorescence and Induction by Mitomycin C

(33258 Hoechst/5-bromodeoxyuridine-dependent fluorescence/isochromatid labeling)

SAMUEL A. LATT*

Clinical Genetics Division, Children's Hospital Medical Center, and Center for Human Genetics and Department of Pediatrics, Harvard Medical School, Boston, Massachusetts

Communicated by Elkan R. Blout, May 28, 1974

ABSTRACT **Sister chromatid exchanges in chromosomes from human lymphocytes grown two replication cycles in medium containing 5-bromodeoxyuridine can be detected by fluorescence microscopy after staining with the bisbenzimidazole dye 33258 Hoechst. These exchanges are much more frequent than chromosome or chromatid breaks and appear to be partly but not entirely due to 5-bromodeoxyuridine incorporation. Sister chromatid exchanges are extremely sensitive indicators of chromosome damage produced by DNA cross-linking agents such as mitomycin C. Significant increases in the sister chromatid exchange frequency occur with 3 ng/ml of mitomycin C; higher concentrations of mitomycin C induce further sister chromatid exchanges. Comparatively few gross chromosomal aberrations are seen in cells exhibiting as many as one hundred or more sister chromatid exchanges. Most of the damage caused by mitomycin C to chromosomal DNA is apparently repaired without detectable changes in chromosome morphology. Analysis of sister chromatid exchanges may permit more sensitive detection of damage to DNA caused by other agents than has previously been possible by classical cytological techniques.**

A microfluorometric method for detection of DNA synthesis and sister chromatid exchanges has recently been developed (1). This method depends on the suppression of fluorescence at neutral pH of the bisbenzimidazole dye 33258 Hoechst when it is bound to chromosome regions containing the base analogue BrdU. Sister chromatid exchanges in cells that have replicated twice in medium containing BrdU are apparent as sharp, reciprocal alterations in fluorescence intensity along metaphase chromosomes. Multiple exchanges in individual chromosomes can be accurately detected over short lengths that might be difficult to resolve by autoradiography, the method previously used to demonstrate sister chromatid exchanges (2).

Sister chromatid exchanges detected in normal human lymphocytes by fluorescence microscopy are at least 200 times more frequent than chromosome and chromatid breaks (3). If these exchanges result from DNA damage that has been repaired, then the predominance of sister chromatid exchanges over breaks indicates that most of the damage occurring in chromosomes would be undetected by techniques able to recognize only gross morphological changes in chromosomes. Chromosome or chromatid breaks and rearrangements, previously used as indices of damage to chromosomes caused by chemicals and irradiation (4–6), might thus represent only a small fraction of the alterations actually occurring in chromosomal DNA. This hypothesis has now been tested by culturing human lymphocytes in the presence of mitomycin C, a DNA cross-linking agent (7).

* Address reprint requests to author at: Children's Hospital Medical Center, 300 Longwood Ave., Boston, Mass. 02115.

MATERIALS AND METHODS

Cell Growth and Slide Preparation. Mitomycin C (Sigma), BrdU (Sigma), or [^{3}H]BrdU, 11 Ci/mole (New England Nuclear Corp.) were added at the beginning of the culture period as described in the *text*. Other aspects of these procedures were as described previously (1, 3).

Staining and Photography. Slides stained with quinacrine for chromosome identification were destained following photomicrography in 3:1 methanol:acetic acid, restained for 10 min with 33258 Hoechst (kindly provided by Dr. H. Loewe, Hoechst AG, Frankfurt am Main, Germany) and photographed as described previously (1, 3). A few slides were stained directly with 33258 Hoechst (1).

Microspectrofluorometry employed a Leitz MPV II instrument with a 40× achromat objective (1). Data points presented represent the average of measurements over approximately 15 different cells. Grain counts after autoradiography were performed on photographs of the same cells used for fluorescence measurement.

RESULTS

Metaphase chromosomes in cells that have replicated twice in medium containing BrdU possess chromatids with markedly different fluorescence intensities when stained with 33258 Hoechst (Fig. 1). This observation is consistent with the semiconservative distribution of newly replicated DNA between sister chromatids (2). The chromatid exhibiting the weaker fluorescence contains BrdU in both chains of its DNA, while that fluorescing more brightly contains BrdU in only one polynucleotide chain (1). Cells that have replicated twice in BrdU can thereby be differentiated from those that have replicated only once, since sister chromatids in the latter type of cell exhibit nearly equal 33258 Hoechst fluorescence. Sharp reciprocal alterations in fluorescence intensity along chromosomes after two cycles of BrdU incorporation signal sister chromatid exchanges†.

The present method for detection of sister chromatid exchanges requires the introduction of the thymidine analogue

† Chromatid segments containing DNA substituted with BrdU in only one polynucleotide chain are apparent as isolated regions of bright fluorescence after a third cycle of replication in BrdU.

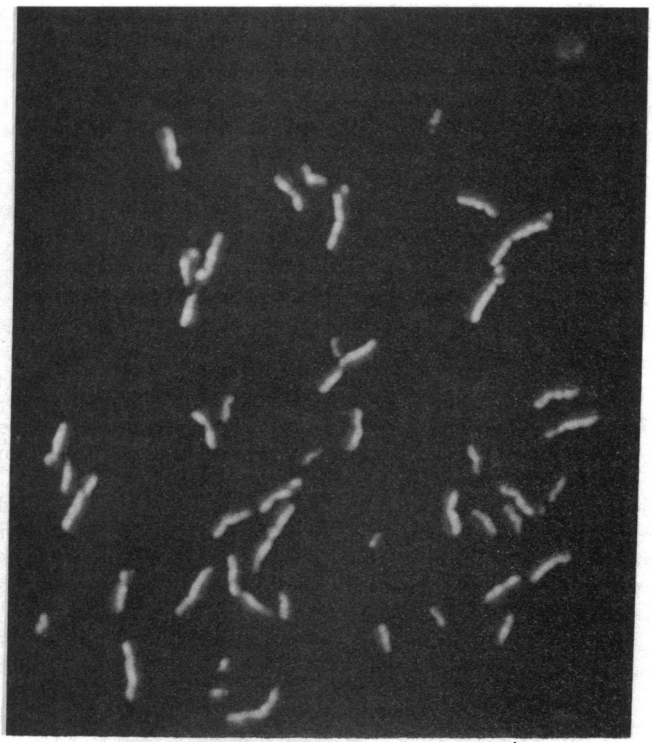

FIG. 1. Detection of sister chromatid exchanges in human metaphase chromosomes by fluorescence microscopy. Peripheral leukocytes from a normal 46, XX human female were grown 73 hr in medium that included 12 μM BrdU. Slides were stained with 33258 Hoechst and cells photographed as described in *Materials and Methods*. Sister chromatid exchanges are evident as sharp reciprocal alterations in fluorescence intensity along chromosomes. There is a total of nine sister chromatid exchanges in the cell shown.

BrdU into chromosomal DNA. Since BrdU has been observed to cause chromosome damage (8, 9), its effect on the sister chromatid exchange frequency was investigated. The minimum concentration of BrdU in the culture medium at which lymphocytes replicating twice can be identified by fluorescence microscopy is about 3 μM, while fluorescence differentiation between sister chromatids sufficient for accurate detection of exchanges requires about 6 μM BrdU. Raising the concentration of BrdU in the culture medium from 6 μM to 24 μM increases the sister chromatid exchange frequency by only 20% (Fig. 2). This higher concentration of BrdU, 24 μM, is sufficient to produce a marked reduction in fluorescence. Employment of fluorescence quenching to monitor the incorporation of nonradioactive BrdU into chromosomes obviates the use of radioactivity, which has been implicated as a cause of sister chromatid exchanges (10–13). At higher BrdU concentrations (47–94 μM) little additional fluorescence quenching is observed. The increase in the sister chromatid exchange frequency between 24 μM and 94 μM BrdU is more than twice that observed over the lower concentration range. The sister chromatid exchange frequency is thus not proportional to fluorescence quenching by BrdU. Instead, the results are compatible with a baseline level of exchanges at very low BrdU concentrations, upon which an increment due to BrdU is superimposed. Subsequent experiments with mitomycin C were performed at 12 μM BrdU.

The use of fluorescence quenching to assess BrdU incorporation was calibrated in a separate experiment employing auto-

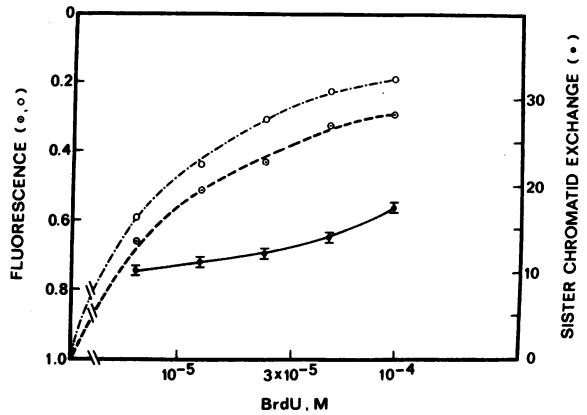

FIG. 2. Dependence of 33258 Hoechst fluorescence and sister chromatid exchange frequency on the concentration of BrdU in the growth medium. Parallel cultures of peripheral leukocytes from a normal 46, XY human male were grown 72 hr in media containing various concentrations of BrdU. After slides were stained with 33258 Hoechst, the fluorescence intensity over 35–50 metaphases per BrdU concentration was determined photometrically. (Note the inverted scale used for fluorescence.) Metaphases were then photographed for identification of the fluorescence patterns corresponding to 1 (⊙) or 2 (○) divisions in BrdU (see *text*). The fluorescence at each concentration of BrdU is presented relative to that of metaphases from control cells not exposed to BrdU. Other cells from these cultures were photographed for enumeration of sister chromatid exchanges (●). Each point represents the average from at least 42 cells. The *vertical bars* indicate the standard error of the mean.

radiography to quantitate tritiated BrdU. The decrease in metaphase fluorescence versus BrdU incorporation after either one or two replication cycles can be described by a single curve (Fig. 3), indicating that the effect of BrdU on dye fluorescence at the degrees of substitution reached is additive, whether the

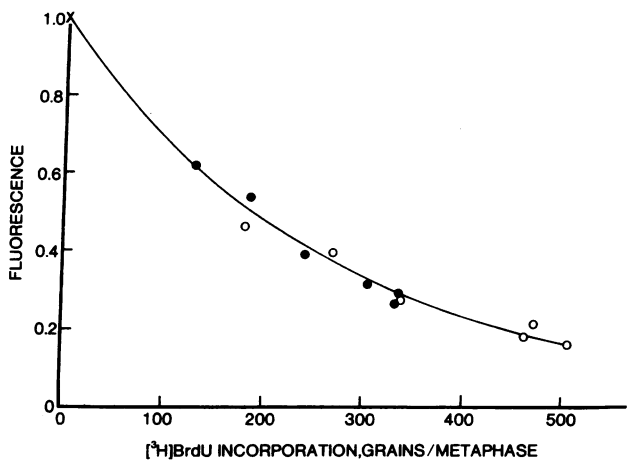

FIG. 3. Dependence of 33258 Hoechst fluorescence intensity on BrdU incorporation into chromosomes. Cells from the same individual as in Fig. 2 were grown as described in that caption except that the BrdU used was tritiated (11 Ci/mole). After fluorescence studies were completed, the slides were processed for autoradiography and grain counts were determined over the same metaphases examined photometrically. Os correspond to cells that replicated twice in medium containing [^{3}H]BrdU, while ●s represent those that replicated only once.

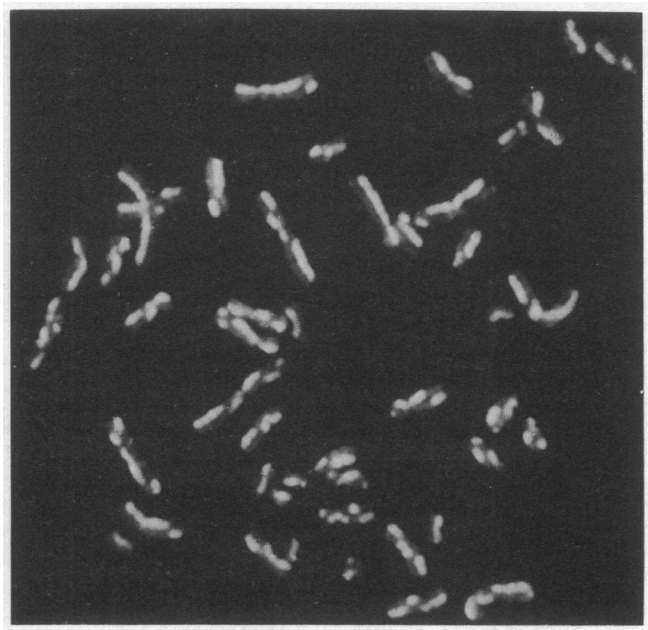

FIG. 4. Sister chromatid exchanges in a cell exposed to mitomycin C. Mitomycin C (30 ng/ml) was added at the inception of a culture otherwise treated identically to that of Fig. 1. A total of 54 sister chromatid exchanges is detected in this cell.

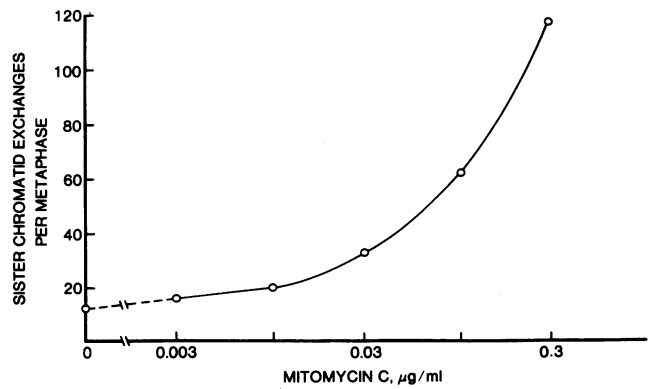

FIG. 5. Dependence of the sister chromatid exchange frequency on mitomycin C concentration. Peripheral leukocytes from a normal 46, XY human male were cultured 74 hr in medium containing 12 μM BrdU and different mitomycin C concentrations. Slides were stained with 33258 Hoechst and photographed as described for detection and tally of sister chromatid exchanges. Except for the data at 0.3 μg/ml of mitomycin C, the *points* represent averages of sister chromatid exchange counts on at least 32 cells. At 0.3 μg/ml of mitomycin C, very few cells replicated twice, and the average shown is based on 10 different cells.

BrdU is present in one or two chains of the DNA. The maximum incorporation of [^{3}H]BrdU into chromosomes obtained after two replication cycles is one and one-half of that after one replication. This accords with the number of DNA chains (three versus two) substituted in the two cases.

An average of 12 sister chromatid exchanges per metaphase occurs in cells that have replicated twice in medium containing 12 μM BrdU. The number of exchanges is increased when the cells are grown in the presence of mitomycin C, a chemical known to cause chromosome breakage (14–16). For example, chromosomes from lymphocytes that have replicated twice in the presence of 30 ng/ml of mitomycin C (Fig. 4) exhibit an average of approximately 40 sister chromatid exchanges but fewer than 0.07 breaks per cell (Table 1). Inhibition of cell replication by mitomycin C is evidenced by an increase in the proportion of metaphases corresponding to cells replicating only once during the 3-day culture period [a time sufficient in the absence of mitomycin C for an average of two replications (17)]. These cells exhibit a much higher frequency of chromosome aberrations, such as breaks and multiradial figures, than do those which have replicated twice. However, the number of aberrations in these cells does not exceed a few percent of the number of sister chromatid exchanges in cells replicating twice at the same mitomycin C concentration.

The frequency of sister chromatid exchanges in human lymphocytes is markedly dependent on the mitomycin C con-

TABLE 1. *Mitomycin C induction of sister chromatid exchanges in lymphocytes of different individuals*

Mitomycin C concentration (ng/ml)	Individuals					
	I	II	III_A	III_B	IV	V
0	11.6 (39) 0	12.2 (60) 0.07	12.1 (32) 0	12.6 (21) 0	9.5 (23) 0	14.6 (28) 0
3	Not done	Not done	16.0 (38) 0.03	14.3 (27) 0	16.5 (43) 0.02	19.0 (26) 0
10	26.0 (25) 0.04	24.3 (55) 0.04	19.6 (37) 0	22.6 (23) 0	23.5 (30) 0.03	29.8 (6) 0
30	49.5 (17) 0.06	Not done	32.4 (36) 0.03	45.7 (29) 0.10	48.2 (36) 0.08	51 (1) 0
100	—*	71 (1) 0	62 (32) 0.06†	112 (2) 0	108 (3) 0	—*

Each roman numeral denotes lymphocytes from a different individual; III_A and III_B refer to two separate experiments with cells from the same individual. The first row of each entry contains the average sister chromatid exchange frequency and, in parentheses, the number of cells contributing to this average. The second row contains the average number of chromosome or chromatid breaks per metaphase in these cells.

* No metaphases corresponding to cells replicating twice were observed.

† One quadriradial figure detected.

centration (Fig. 5). A statistically significant increase in exchanges is produced by as little as 3 ng/ml of this drug (Table 1)‡. At 100–300 ng/ml, the exchange frequency can exceed 100 per cell.

Very high sister chromatid exchange frequencies probably require optical detection methods for accurate analysis. Even when exchanges are closely spaced on individual chromosomes, the changes in fluorescence along chromatids are reciprocal (Fig. 6). Autoradiography of chromosomes such as those shown (Fig. 6) might not resolve some of the closely spaced exchanges but would exhibit grains over both chromatids at the same location; a distribution interpreted as reflecting isochromatid labeling (18). A similar explanation for isochromatid labeling, based on the resolution limit of autoradiography employing light microscopy, has recently been proposed after statistical considerations (19).

The spatial distribution of mitomycin C-induced sister chromatid exchanges resembles the distribution both of mitomycin C-induced breaks (16) and of sister chromatid exchanges occurring in the absence of mitomycin C (3). The frequency of these exchanges in individual chromosomes generally increases with metaphase chromosome length. Within a given chromosome, the majority of exchanges appear to be either in interband regions [defined by quinacrine banding patterns (20)] or very near band–interband junctions. Unlike the translocations and quadriradial figures induced by mitomycin C (14–16), the sister chromatid exchanges are not preferentially located on chromosomes no. 1, no. 9, and no. 16.

Small variations in the numbers of sister chromatid exchanges induced by a particular concentration of mitomycin C have been observed in different experiments (Table 1). Differences both in culture conditions and in the effectiveness of chromosome repair in individuals might contribute to these results.

DISCUSSION

Sister chromatid exchanges, detected by fluorescence microscopy, are sensitive indicators of the impact of mitomycin C on human chromosomes. Mitomycin C was chosen for this initial investigation because its effect on cellular DNA is marked and relatively well characterized (11, 14, 15, 16, 21). However, the approach described should have wide applicability. Sister chromatid exchanges are much more frequent than unrepaired breaks, and contain information about both chromosome damage and repair. Coordinate analysis of breaks and exchanges should constitute a convenient method for distinguishing alterations in these processes due to external agents and disease states.

Use of 33258 Hoechst fluorescence to detect sister chromatid exchanges requires the incorporation of a nucleotide analogue such as BrdU into chromosomal DNA. BrdU has been known to promote chromosome damage, either directly or by photosensitization (8, 9, 22), and in some systems it has also been observed to affect DNA repair (23). The observed dependence of sister chromatid exchanges on BrdU incorporation (Figs. 2 and 3) suggests that low doses of the analogue might have little effect on the exchange frequency. However, if BrdU were to increase the number of sister chromatid exchanges induced by exposure to another agent, it would in effect serve to enhance the sensitivity of the system for detecting such additional damage.

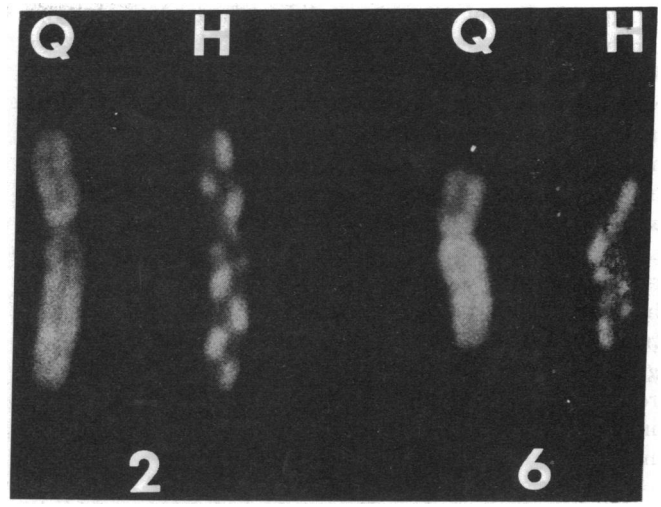

FIG. 6. Multiple sister chromatid exchanges in individual chromosomes induced by mitomycin C. The chromosomes shown (no. 2 on the *left*, no. 6 on the *right*) are from a cell grown in medium containing 10 μM BrdU and 0.3 μg/ml of mitomycin C. They were stained with quinacrine for identification (*Q, left hand* member of each pair) and subsequently with 33258 Hoechst (*H, right hand* member of each pair). A large number of sister chromatid exchanges is observed in these two chromosomes. The quinacrine fluorescence outlines individual chromatids, aiding in the differentiation of sister chromatid exchanges from occasional 180° twists of chromatid arms. Changes in fluorescence intensity are reciprocal at each exchange point. The actual lengths of the chromosomes shown are 12 μm (no. 2) and 8 μm (no. 6).

The present method requires growth of cells for at least one replication cycle in medium containing BrdU followed by a second replication during which the presence of the analogue is optional. If the agent to be tested is present during the entire culture period, the results are subject to selective forces (24) which are manifest as a reduction in the average number of divisions by severely damaged cells. However, cells that undergo different numbers of replication cycles in BrdU can be distinguished by their characteristic 33258 Hoechst metaphase chromosome fluorescence patterns. Alternatively, an agent to be studied can be added for a limited interval prior to cell harvest.

An increase in the frequency of sister chromatid exchanges has been detected in cells exposed for 3 days to as little as 0.003 μg/ml of mitomycin C (Fig. 5, Table 1). In contrast, the concentrations of this agent required to generate large numbers of breaks range from 0.1 to 1.0 μg/ml (14–16). In some of these studies, exposure to the drug was less than 3 days, but the differences in duration of exposure are much less than the differences in mitomycin C concentration employed in the two approaches. The relatively high frequency of sister chromatid exchanges, compared with chromosome or chromatid breaks, permits data on DNA damage to be acquired by examination of exchanges in fewer cells than is necessary for a comparable analysis of breaks. Other agents previously examined for their ability to cause gross chromosome structural aberrations might be re-examined for their ability to alter the frequency of sister chromatid exchanges in human cells.

‡ In the four experiments in which the effect of 3 ng/ml mitomycin C was examined, the average number of sister chromatid exchanges per cell was 16.5 (144 cells, standard error of the mean = 0.4), compared with a control value of 12.3 (108 cells, standard error of the mean = 0.5).

The induction of sister chromatid exchanges by mitomycin C may reflect the action of basic cellular DNA repair processes. Mitomycin C is known to alkylate DNA (24) and crosslink complementary polynucleotide chains (7). Mitomycin C-induced crosslinks, as well as those introduced by nitrogen mustards and by a furocoumarin derivative, 4,5′,8-trimethylpsoralen, in conjunction with light, are normally excised during an apparently complicated repair process (25). Agents that cross-link DNA have also been observed to stimulate genetic recombination (25, 26) as well as chromatid exchanges between homologous chromosomes (27). The chemical steps in the repair of cross-linked DNA have been studied in especially great detail in the case of the trimethylpsoralen-plus-light reaction. Repair of these cross-links in bacteria appears to involve first incision and then excision of regions of one polynucleotide chain involved in a crosslink, followed by exchange of polynucleotide chains between homologous DNA molecules (25). The possibility that such an exchange mechanism is operative in mammalian cells in response to removal of cross-links has been suggested (25). The present observation of increased numbers of sister chromatid exchanges in human lymphocytes exposed to mitomycin C supports this hypothesis.

Sister chromatid exchanges are produced by a number of agents that damage DNA in addition to mitomycin C. We have recently observed that the nitrogen mustard mechlorethamine, another chemical that cross-links DNA, promotes sister chromatid exchanges at concentrations as low as 4 ng/ml. In addition, ultraviolet light, which does not cross-link DNA but produces thymidine dimers and other photoproducts (22, 23), increases the exchange frequency (28), and, in cells of other organisms, promotes genetic recombination (26). Irradiation of cells with x-rays has also been observed to increase the sister chromatid exchange frequency (29). This latter treatment is known to break DNA chains (30, 31). The processes by which cross-links, thymine dimers, and x-ray-induced breaks are repaired are all thought to include an intermediate state in which damaged regions of one polynucleotide chain have been removed but not completely replaced (25, 32). Perhaps many of the exogenously induced sister chromatid exchanges occur in such a state of partial DNA repair. If this is in fact true, then sister chromatid exchanges might be a constant feature of DNA repair processes, making the present method for their detection one of general utility in monitoring DNA damage in human chromosomes. Conversely, elucidation of the precise stage at which these exchanges are introduced may shed light on the fundamental processes of repair common to different types of DNA damage, and perhaps to genetic recombination as well. Such knowledge may also serve to increase understanding of disease states, including those predisposing to chromosome fragility and associated with neoplasia (33).

The continued expert technical assistance of Miss Lois Juergens and advice and support of Dr. Park S. Gerald are gratefully acknowledged. This work was supported by grants from the National Institute of Child Health and Human Development (HD-06276), National Institute of General Medical Sciences (GM-21121), and the American Cancer Society, Massachusetts Division, Inc.

1. Latt, S. A. (1973) *Proc. Nat. Acad. Sci. USA* **43,** 122–138.
2. Taylor, J. H. Woods, P. S. & Hughes, W. L. (1957) *Proc. Nat. Acad. Sci. USA* **43,** 122–138.
3. Latt, S. A. (1974) *Science* **185,** 74–76.
4. Evans, H. J. (1962) *Int. Rev. Cytol.* **13,** 221–321.
5. Kihlman, B. A. (1971) *Advan. Mol. Biol.* **1,** 59–107.
6. Buckton, K. E. & Evans, H. J. (1973) *Methods for the Analysis of Human Chromosome Aberrations* (World Health Organization, Switzerland) 1–66.
7. Iyer, V. N. & Szybalski, W. (1963) *Proc. Nat. Acad. Sci. USA* **50,** 355–362.
8. Hsu, T. C. & Somers, C. E. (1961) *Proc. Nat. Acad. Sci. USA* **47,** 396–403.
9. Chu, E. H. Y. (1965) *Mutat. Res.* **2,** 75–94.
10. Taylor, J. H. (1958) *Genetics* **47,** 514–527.
11. Wolff, S. (1964) *Mutat. Res.* **1,** 337–343.
12. Marin, G. & Prescott, D. M. (1964) *J. Cell Biol.* **21,** 159–167.
13. Gibson, D. A. & Prescott, D. M. (1972) *Exp. Cell Res.* **74,** 397–402.
14. Nowell, P. C. (1964) *Exp. Cell Res.* **33,** 445–449.
15. Shaw, M. W. & Cohen, M. M. (1965) *Genetics* **51,** 181–190.
16. Morad, M., Jonasson, J. & Lindsten, J. (1973) *Hereditas* **74,** 273–282.
17. Sasaki, M. S. & Norman, A. (1966 *Nature* **212,** 913–914.
18. Comings, D. E. (1972) *Advan. Hum. Genet.* **3,** 237–431.
19. Gibson, D. A. & Prescott, D. M. (1973) *Exp. Cell Res.* **83,** 445–447.
20. Caspersson, T., Lindsten, J., Lomakka, G., Moller, A. & Zech, L. (1972) *Int. Rev. Exp. Pathol.* **11,** 1–72.
21. Weissbach, A. & Lisio, A. (1965) *Biochemistry* **4,** 196–200.
22. Elkind, M. M. & Whitmore, G. F. (1967) *The Radiobiology of Cultured Mammalian Cells* (Gordon and Breach, New York, London, Paris).
23. Setlow, R. B. & Setlow, J. K. (1972) *Annu. Rev. Biophys. Bioeng.* **1,** 293–346.
24. Sasaki, M. S. & Norman, A. (1967) *Nature* **214,** 502–503.
25. Cole, R. S. (1973) *Proc. Nat. Acad. Sci. USA* **70,** 1064–1068.
26. Howard-Flanders, P. & Lin, P. F. (1973) *Genetics* Suppl. **73,** 85–90.
27. German, J. & La Rock, J. (1969) *Tex. Rep. Biol. Med.* **27,** 409–418.
28. Kato, H. (1973) *Exp. Cell Res.* **82,** 383–390.
29. Gatti, M. & Olivieri, G. (1973) *Mutat. Res.* **17,** 101–112.
30. Lett, J. T., Caldwell, I., Dean, C. J. & Alexander, P. (1967) *Nature* **214,** 790–792.
31. Freifelder, D. (1968) *J. Mol. Biol.* **35,** 303–309.
32. Cleaver, J. E. (1969) *Proc. Nat. Acad. Sci. USA* **63,** 428–435.
33. German, J. (1972) *Progr. Med. Genet.* **8,** 61–101.