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SISTER CHROMATID EXCHANGE FREQUENCIES WITHIN HOMOGENEOUSLY STAINING REGIONS OF A METHOTREXATE-RESISTANT MURINE CELL LINE

THE UNIVERSITY OF ARIZONA M.S. 1983

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SISTER CHROMATID EXCHANGE FREQUENCIES WITHIN HOMOGENEOUSLY STAINING REGIONS OF A METHOTREXATE-RESISTANT MURINE CELL LINE

by

Rebecca Dee Broderick

A Thesis Submitted to the Faculty of the

COMMITTEE ON GENETICS (GRADUATE)

In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE

In the Graduate College

THE UNIVERSITY OF ARIZONA

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STATEMENT BY THE AUTHOR

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6/15/83
Date

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ABSTRACT

Two murine lymphoblastoid cell lines, L5178YR and L5178YS, were cultured in media containing the thymidine analog 5-bromodeoxyuridine (BrdU). Two rounds of DNA replication followed by treatment with the fluorochrome 33258 Hoechst and counterstaining with Giesma produced differential chromatid staining. The frequency of sister chromatid exchange (SCE) was recorded for 50 metaphases from each cell line, and chromosome lengths of each metaphase were measured. From these measurements, the SCE/unit length (SCE/U.L.) was calculated. The methotrexate-resistant (MTX^R) cell line did not have a statistically significant difference ($p = 0.251$) in SCE/U.L. when compared to its methotrexate-sensitive (MTX^S) parental line. Additionally, in L5178YR the SCE/U.L. of the homogeneously staining regions (HSRs) was statistically identical to the remainder of the L5178YR genome, ($p > 0.05$) and their distribution was random according to Poisson expectations ($p > 0.20$). The results strongly suggest that SCEs occur at the same frequency in HSR-bearing chromosomes as in the remainder of the resistant genome.

INTRODUCTION

Using the English broad bean Vicia faba, Taylor, Woods, and Hughes (1957), first demonstrated the differential labeling of sister chromatids. Seedlings were allowed to grow in a solution containing tritiated thymidine (H^3-TdR) for one round of DNA replication, followed by transfer of seedlings to a non-radioactive solution containing colchicine for additional rounds of DNA replication. Subsequently, harvesting of root tip material for metaphase cells was performed. After autoradiography, cells with 4N chromosome numbers (those cells which had completed two rounds of DNA replication in the presence of the label) consisted entirely of labeled chromosomes. However, only one sister chromatid of each chromosome appeared to have incorporated the radioactive label, a finding consistent with the semiconservative model of DNA replication (Meselson and Stahl, 1958). In some instances a chromatid was labeled only part of its entire length, and the complementary portion of the adjacent unlabeled chromatid was labeled. This phenomenon was attributed to physical exchange between homologous chromatids, termed "sister chromatid exchange" (SCE).

Additional investigations by Taylor (1958) and Prescott and Bender (1963) produced similar results which verified the theory of semiconservative replication of DNA. However, apparent inconsistencies, such as isolabeling of both chromatids, frequently

were observed. This issue was resolved when Latt (1973) developed a technique which allowed fluorometric documentation of the semiconservative distribution of newly replicated DNA between sister chromatids and the detection of sister chromatid exchanges. In this method, incorporation of the thymidine analog, 5-bromodeoxyuridine (BrdU), replaced the use of H^3-TdR. Thus, instead of an up to one month delay for development of chromosome preparations using H^3-TdR, chromosome preparations which had incorporated BrdU could be treated with the fluorochrome compound 33258 Hoechst and examined for differential staining immediately. This fluorescent method also provided greater precision in SCE detection by eliminating the use of radioactive nucleotides, thus eliminating the inconsistencies caused by variations in path length of β-particle decay often observed in autoradiographic preparations.

Even though the method of Latt provided a distinct advantage over previous radiolabeling techniques, the fluorescent images faded rapidly. A superior technique producing permanent preparations of differentially labeled sister chromatids was developed in 1974 by Perry and Wolff using fluorescence plus Giesma (FPG). BrdU substituted chromosomes were stained with the fluorochrome 33258 Hoechst, followed by incubation in phosphate buffer and counterstaining with Giesma. Cells which pass through their first S phase in the presence of BrdU contain unifilarly substituted chromatids and stain uniformly dark. After a second division in the presence of BrdU, metaphase chromosomes appear harlequin; the unifilarly substituted chromatids stain darkly while the bifilarly

substituted chromatids stain lightly (Figure 1). It is from these second division cells that SCEs are scored (Figure 2). With each succeeding cell generation, the percentage of darkly staining chromatids decreases by one half. Thus, the number of mitotic divisions each cell had undergone can easily be determined by this technique (Figure 3).

In this thesis, SCE was used to examine the properties of a particular chromosomal aberration frequently found in drug resistant mammalian cells. The presence of characteristic chromosomal abnormality accompanying the phenotypic conversion to drug resistance in methotrexate-resistant (MTX R) mammalian cells was first observed by Biedler and Spengler in 1976. They described long, intermediate staining areas on some metaphase chromosomes following G-banding and called them "homogeneously staining regions" (HSRs). Since their initial discovery, HSRs have been reported in a large number of drug resistant cell lines and a variety of different tumor cell populations (Balaban-Malenbaum and Gilbert 1977, Quinn et al. 1979, Trent 1980, Barker et al. 1980, Biedler et al. 1980, Balaban and Gilbert 1982, Trent 1982).

L5178YR, the MTX R murine lymphoblastoid cell line used in this study, contains two marker chromosomes which bear HSRs (Figure 1). The mechanism of MTX resistance in L5178YR results from increased production of the target enzyme dihydrofolate reductase (DHFR). DHFR is increased up to 200 fold, enabling the MTX R L5178YR cells to survive in a dosage of MTX 10,000 times greater than that of the control (Dolnick et al. 1979).

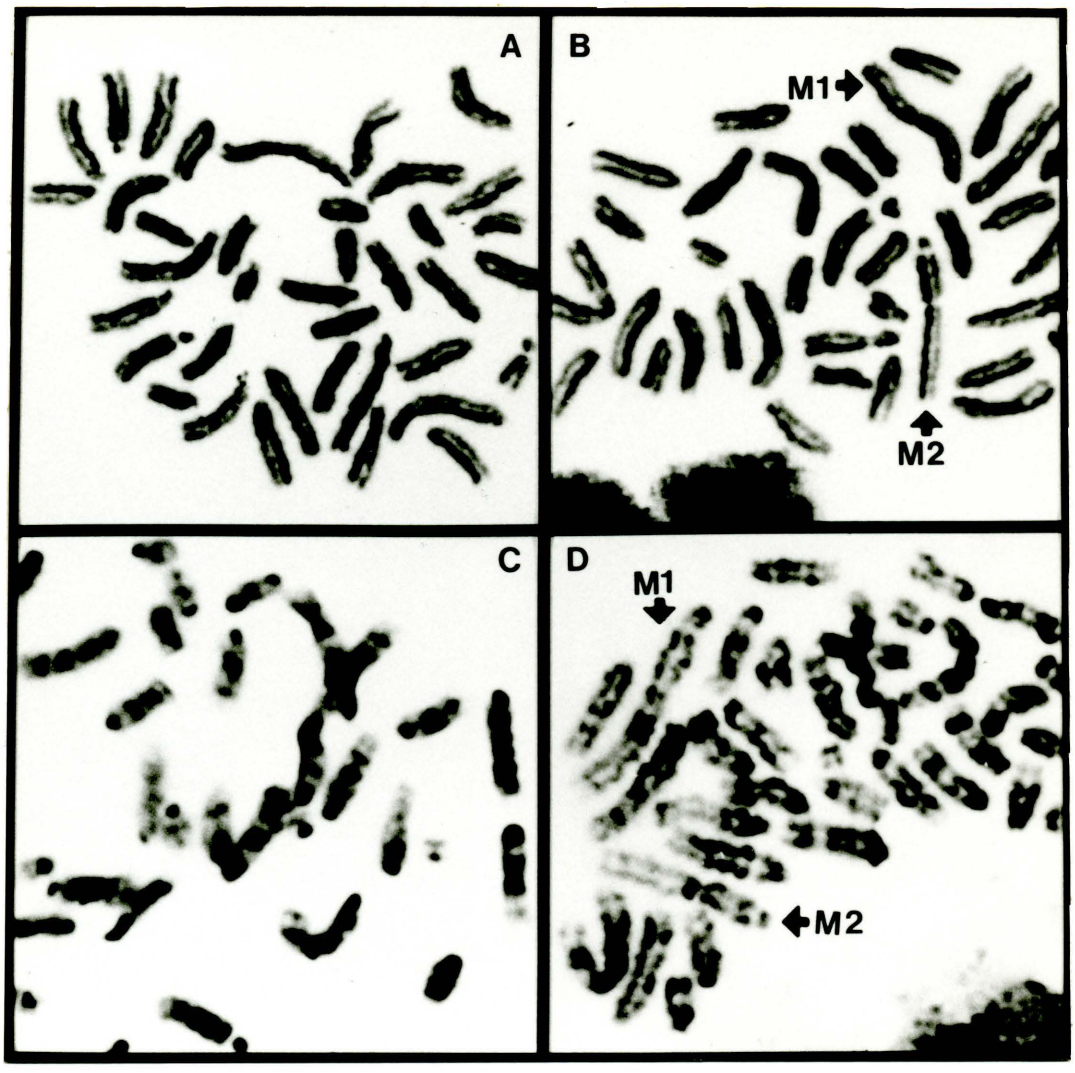

Figure 1. Staining patterns of L5178YS and L5178YR.

(A) Fluorescence plus Giesma (FPG) stained L5178YS metaphase. (B) Fluorescence plus Giesma (FPG) stained L5178YR metaphase. (C) G-banded L5178YS partial metaphase. (D) G-banded L5178YR partial metaphase.

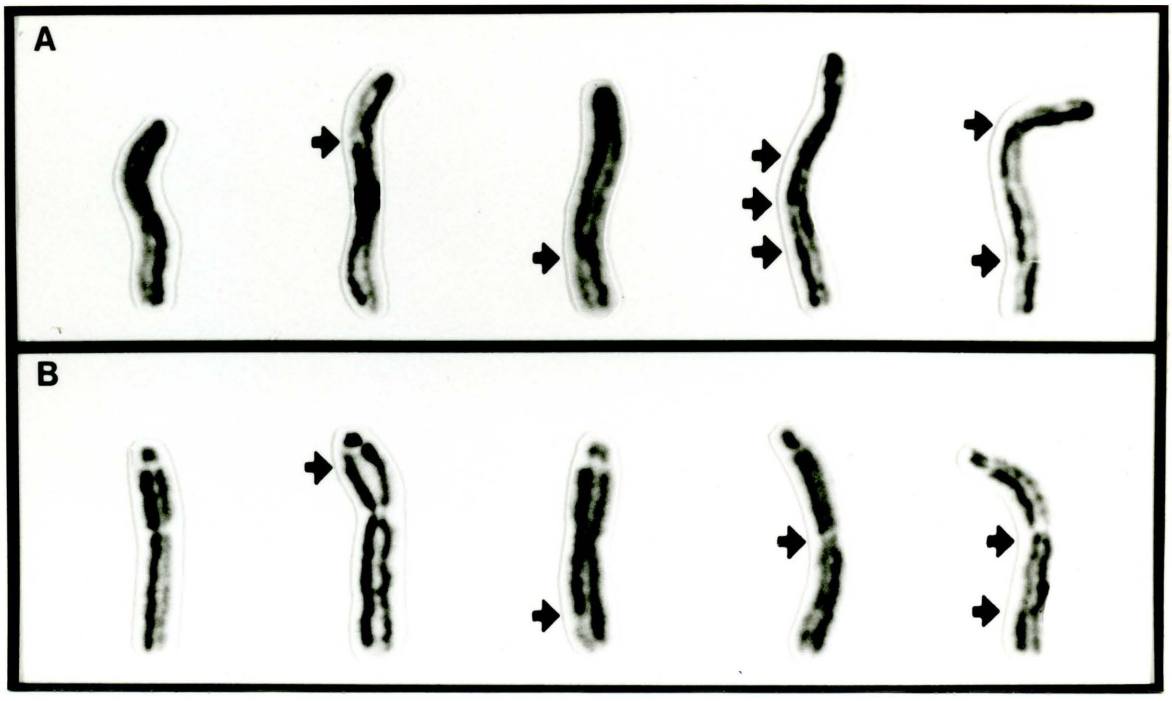

Figure 2. SCE along HSR-bearing markers.

(A) HSR-bearing marker 1 from L5178YR, arrows represent examples of SCE. (B) HSR-bearing marker 2 from L5178YR, arrows represent examples of SCE.

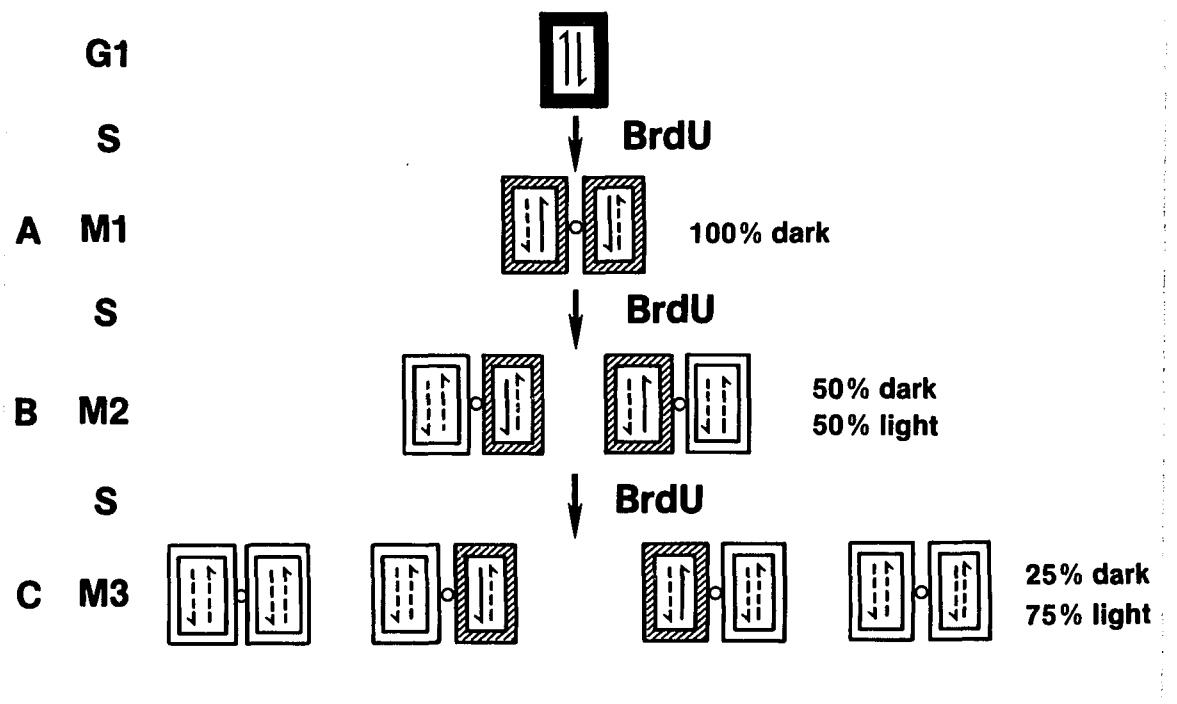

Figure 3. BrdU differential labeling.

(A) After the first round of DNA replication in the presence of BrdU, each chromatid is unifilarly substituted and stains darkly. (B) A second round of DNA replication in the presence of BrdU produces differentially labeled chromosomes when stained with FPG. The unifilarly substituted chromatid stains darkly while the bifilarly substituted chromatid stains lightly. (C) With each successive round of replication in the presence of BrdU, the percent of chromatids staining darkly would ideally be reduced by 50 percent.

In situ hybridization studies using radiolabeled recombinant plasmids containing cDNA to DHFR mRNA revealed that the amplified DHFR genes are located within the two HSRs of the MTX ^{R} L5178YR cell line (Trent 1982). HSRs in this system and others have been shown to represent the cytological manifestation of somatic gene amplification (Balaban-Malenbaum and Gilbert 1980, Harper and Kellems 1981, Schimke 1982).

One theory proposed to account for gene amplification within the MTX ^{R} cells is unequal crossing over between sister chromatids (Wolff 1979, Schimke 1982, German 1982). This mechanism would result in a heterogeneous population, some cells with fewer gene copies, others possessing greater numbers of gene copies. Those MTX ^{R}cells with increased copies of DHFR presumably would be selected following exposure to increasing concentrations of MTX (Schimke et al. 1978, Schimke et al. 1981). Consequently, continued episodes of unequal SCE have been proposed as one possible mechanism of gene amplification resulting in HSR formation. A second finding which is potentially related to a mechanism of unequal SCE is the observation that SCE may be especially prevalent at or near the DHFR locus (Chasin et al. 1982).

This thesis was designed to determine if SCE frequencies along the length of the HSR-bearing marker chromosomes of L5178YR demonstrated altered frequencies of SCE/U.L. when compared to the remaining L5178YR genome, and the L5178YS parental cell line. The frequency of SCE/U.L. was calculated for L5178YR and L5178YS. Additionally, the expected frequency of SCE/U.L. was

determined from the non-HSR-bearing chromosomes of L5178YR and compared to the observed frequency of SCE/U.L. in the HSR-bearing markers. Also, Poisson expectations were used to determine if SCE distribution among the HSR-bearing markers was random. Finally, the distribution of SCE along the HSR-bearing markers was examined to ascertain if their occurrence was random or limited to specific regions of the chromosomes.

MATERIALS AND METHODS

Cell Culturing

The murine lymphoblastoid cell lines L5178YS and L5178YR (a generous gift of R.T. Schimke, Professor and Head, Department of Biology, Stanford University) were cultured in M5 media composed of equal volumes of Dulbecco's MEM and Ham's F12 medium supplemented with 2.0 gm/l $NaHCO_3$, 20.0 µg/ml insulin, 5.0 µg/ml transferrin, 15 mM Hepes, 10^{-7} M hydrocortisone, 100 units/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml fungizone, and 10 percent horse serum. L5178YR cells received 5.0 µg/ml of methotrexate (MTX). Cultures were incubated at 37°C in a 5 percent CO_2 incubator.

BrdU Labeling and Cell Harvesting

5.0 µg/ml of 5-bromodeoxyuridine (BrdU) was added to the cultures at initiation. During an incubation period of 24 hours, the flasks were wrapped in aluminum foil and kept in the dark to minimize photolysis of the BrdU-substituted DNA.

Cells were arrested at metaphase by adding colchicine 45 minutes prior to each harvest at a final concentration of 1×10^{-7} M. Cultures were transferred to 15 ml conical tubes and centrifuged for 10 minutes at 1250 rpm in a Sorvall table top centrifuge. The supernatant was decanted and the pellet resuspended in 0.075 M KCl at

37°C. Cultures were incubated an additional 7 minutes in hypotonic at 37°C. Following incubation, the cultures were recentrifuged for 10 minutes and the supernatant decanted. Cold fixative (3:1 absolute methanol:glacial acetic acid) was added slowly to the pellet. The suspensions were mixed with a vortex and stored at -9°C for a minimum of one hour. Cell suspensions were washed twice more with fixative. After the final wash, the supernatant was decanted and the pellet resuspended in 0.5 mls of fixative.

Slide Preparation and Staining

Cell suspensions were mixed, and while holding the slides over boiling water, 6-8 drops of the suspensions were dropped onto pre-cleaned slides. A sharp burst of air was delivered to each slide in order to disperse the cells. Slides were then dried in a 57°C oven for a minimun of 24 hours. After aging slides for 72 hours, they were stained with 33258 Hoechst (100 μg/ml) for 10 minutes, rinsed in distilled water, mounted in 0.6 M Na_2HPO_4, and covered with a 22 x 40 mm coverslip. Slides were then placed in clear containers, exposed to sunlight for 20-35 minutes, and incubated for 5 minutes in phosphate buffer (equal volumes of Na_2HPO_4 and KH_2PO_4 at pH 6.8) at 56°C, followed by counterstaining in 4 percent Giesma (Harleco).

Photography

Slides from 24-hour harvests of L5178YS and L5178YR cultures were scanned up and down from left to right in order to identify and

locate second division metaphases. Metaphases were selected for photography if they contained no or very few overlaps and if the staining was consistant throughout. Additionally, L5178YR metaphases were selected for photography only if they contained one or both of the two possible marker chromosomes.

Under oil immersion, selected L5178YR and L5178YS metaphases were photographed using a Zeiss Universal II photomicroscope. Kodak Technical Pan film at a DIN setting of 14 or 15 was used with a final optical magnification of 1008X.

Following photography, negatives were developed 8 minutes at 20^{o}C using Kodak HC110 developer (dilution F: 1 part HC110 to 19 parts of distilled water). After three water rinses, Kodak fixative was added for 3 minutes and discarded, followed by a distilled water rinse. Kodak hypoclear (1 part Kodak Hypoclear to 4 parts of distilled water) was then added for two minutes. Negatives were rinsed for 5 minutes in running water, rinsed in distilled water, and treated with Kodak Photo-Flo. Negatives were air dried.

Prints of metaphases were made using an automatic print processer. Kodabrome II RC N2 and N3 papers were selected to achieve the appropriate contrast. Enlarger exposure times of 8 to 25 seconds were used depending on the contrast of the negatives. After exposure, prints were processed, fixed for 5 minutes, rinsed for 5 minutes in running water, and allowed to air dry.

Data Analysis and Statistical Methodology

Fifty second division metaphases were photographed from both L5178YS and L5178YR cultures. From these photographs, relative length measurements were made of the following three parameters: 1) the mean total chromosome length of L5178YR cells, 2) the mean total chromosome length of L5178YS cells, and 3) the mean total chromosome length of the HSR-bearing markers M1 and M2 in the L5178YR cells. All measurements were made using a Zeiss MOP 3 Image Analyzer. SCE/U.L. was determined for L5178YS and L5178YR cells with the frequency for each cell line compared using Students t Test. The mean total chromosome length of each cell line was also compared using Student's t Test as well as the mean values of SCE/cell in the lines.

The percent of the total chromosomal length comprised by the HSR-bearing markers was calculated for 50 cells from the L5178YR cell line. This proportion was multiplied by the frequency of SCE/U.L. in the non-HSR-bearing chromosomes in order to determine the expected frequency of SCE/U.L. in the HSRs. Chi-square analysis was then used to determine if the frequency of SCE/U.L. in the HSRs differed from the expected distribution.

The occurrence of SCE within the HSR-bearing markers was examined with chi-square analysis to determine if SCE distribution was random or limited to a specific area(s) of the markers. Each of the two marker chromosomes was divided into five regions of equal length. The frequency of SCE was recorded for each region of the

markers. The expected frequency of SCE for each region was calculated by dividing the total number of exchanges occurring on the markers by five. From these observed and expected values, chi-square analysis was performed at four degrees of freedom.

RESULTS AND DISCUSSION

Distribution of SCE Between Cell Lines

The total lengths of the fifty cells from L5178YS and L5178YR were measured and compared using Student's t Test. The L5178YR cells demonstrated a significantly greater average chromosome length ($p = 0.004$), suggesting that the resistant cells contain more DNA than their sensitive counterparts. When the lengths of L5178YS cells were compared to the non-HSR-bearing component of L5178YR cells (Table 1), there was no significant difference ($p = 0.983$). This observation, when combined with the aforementioned evidence of a greater than 200 fold DHFR amplification in the MTX ^{R} L5178YR cells, suggests that the increased total chromosome length of the L5178YR cell line results from DNA sequence amplification associated with the two HSR-bearing marker chromosomes.

Student's t Test was used to compare the frequency of SCE/cell in the two cell lines, with the results presented in Table 2. SCE/cell was significantly greater in L5178YR when compared to L5178YS ($p = 0.028$). However, when SCE/cell frequencies were compared between L5178YS and the non-HSR-bearing component of L5178YR, there was no significant difference ($p = 0.153$). Because in human and other mammalian chromosomes the distribution

Table 1. Student's t Test comparing total lengths.

	L5178YR	L5178YS	L5178YR-(M1 + M2)
N	50	50	50
$\overline{X}$	548.15	503.26	502.99
sd	81.06	68.43	76.47
t stat.	t = 2.99		t = 0.0187
df = 98	p = 0.004		p = 0.983

Table 2. Student's t Test comparing SCE/cell.

	L5178YR	L5178YS	L5178YR-(M1 + M2)
N	50	50	50
$\overline{X}$	8.88	7.60	8.44
sd	2.95	2.85	3.04
t stat.	t = 2.21		t = 1.42
df = 98	p = 0.028		p = 0.153

of SCEs among chromosomes is generally proportional to chromosome length or DNA content (Ikushima and Wolff 1974, Latt 1974, Morgan and Crossen 1977), the increased chromosome length of L5178YR cells appear to account for the observed increased frequency in SCE between these cell lines. This reasoning is further supported when SCE/U.L. for L5178YS and L5178YR cell lines are compared.

The value of SCE/U.L. of chromosome was calculated for each of the 50 cells analyzed from L5178YS and L5178YR. Additionally, in all L5178YR cells, SCE/U.L. of the HSRs was defined and subtracted from the non-HSR-bearing chromosomes. Using Student's t Test, SCE/U.L. was compared for L5178YS and L5178YR (Table 3) demonstrating no significant difference in SCE/U.L. ($p = 0.251$). A comparison between L5178YS and L5178YR excluding its markers also proved statistically insignificant ($p = 0.116$, Table 3).

Additionally, the frequency of SCE/U.L. in the HSR-bearing markers M1 and M2 was compared to the expected frequency of SCE/U.L. for this region based on the SCE occurrence in the remainder of the L5178YR genome. This result suggested that the total frequency of SCE/U.L. for the HSR-bearing markers did not differ significantly from the expected value based on a similar chromosome length of L5178YR chromosomes ($p > 0.05$, Table 4). Poisson expectations were also used to examine the occurrence of SCEs in the HSR-bearing markers. Again the results were not significant ($p > 0.20$, Table 5).

Finally, to test the hypothesis that SCE may be limited to a specific area(s) of the HSR-bearing marker chromosomes, the markers M1 and M2 were divided longitudinally into five regions of equal

Table 3. Student's t Test comparing SCE/U.L.

	L5178YR	L5178YS	L5178YR-(M1 + M2)
N	50	50	50
$\bar{X}$	0.0167	0.0153	0.0173
sd	0.0065	0.0056	0.0073
t stat.	t = 1.151		t = 1.566
df = 98	p = 0.251		p = 0.116

Table 4. Chi-square analysis of SCE frequency in non-markers.

$$\text{expected SCE frequency} = \frac{\text{L5178YR marker length}}{\text{L5178YR length - markers}} \times \text{SCE frequency in non-markers}$$

Total X^2	df	Probability
65.64	49	$p > 0.05$

Table 5. Poisson distribution of SCEs.

Number of HSR-bearing markers with indicated number of SCEs				
	0	1	2	3
OBS.	34	10	5	1
EXP.	31.55	14.50	3.35	0.50
	χ^2	df	probability	
	2.90	2	p >0.20	

length. Chi-square analysis (Figure 4) demonstrated no significant deviation from the expected random distribution of SCE along either marker (M1: $p > 0.50$, M2: $p > 0.70$).

Mechanisms of HSR Formation and SCE

Although the biochemical and molecular mechanisms of HSR formation are unknown, it has been theorized that unequal crossing over may play a role in their formation (Wolff 1979, Schimke 1982, German 1982). If SCE frequencies are selectively increased in a specific chromosomal region, then the possibility of unequal SCE also rises. Consequently, it is possible that a correlation may exist between SCEs and HSRs. Three previous studies have examined this question. The first reported that SCEs were reduced within HSRs of human neuroblastoma cells (Balaban-Malenbaum and Gilbert 1978), while two other studies found no significant difference in SCE/U.L. within HSR-bearing markers when compared to the remainder of their genomes (George and Powers 1980, Chasin et al. 1982). Unfortunately, all three of these studies were meeting abstracts and fail to provide any statistical treatment of their observations. Therefore, this study was designed to determine whether SCE frequencies differed between the HSR-bearing marker chromosomes of L5178YR when compared to the remainder of the genome.

As previously mentioned, gene amplification and HSR formation have been suggested to occur via unequal crossing over. Accordingly, SCE may be elevated at specific chromosomal areas (i.e., HSRs), while other areas have normal or decreased levels. If this

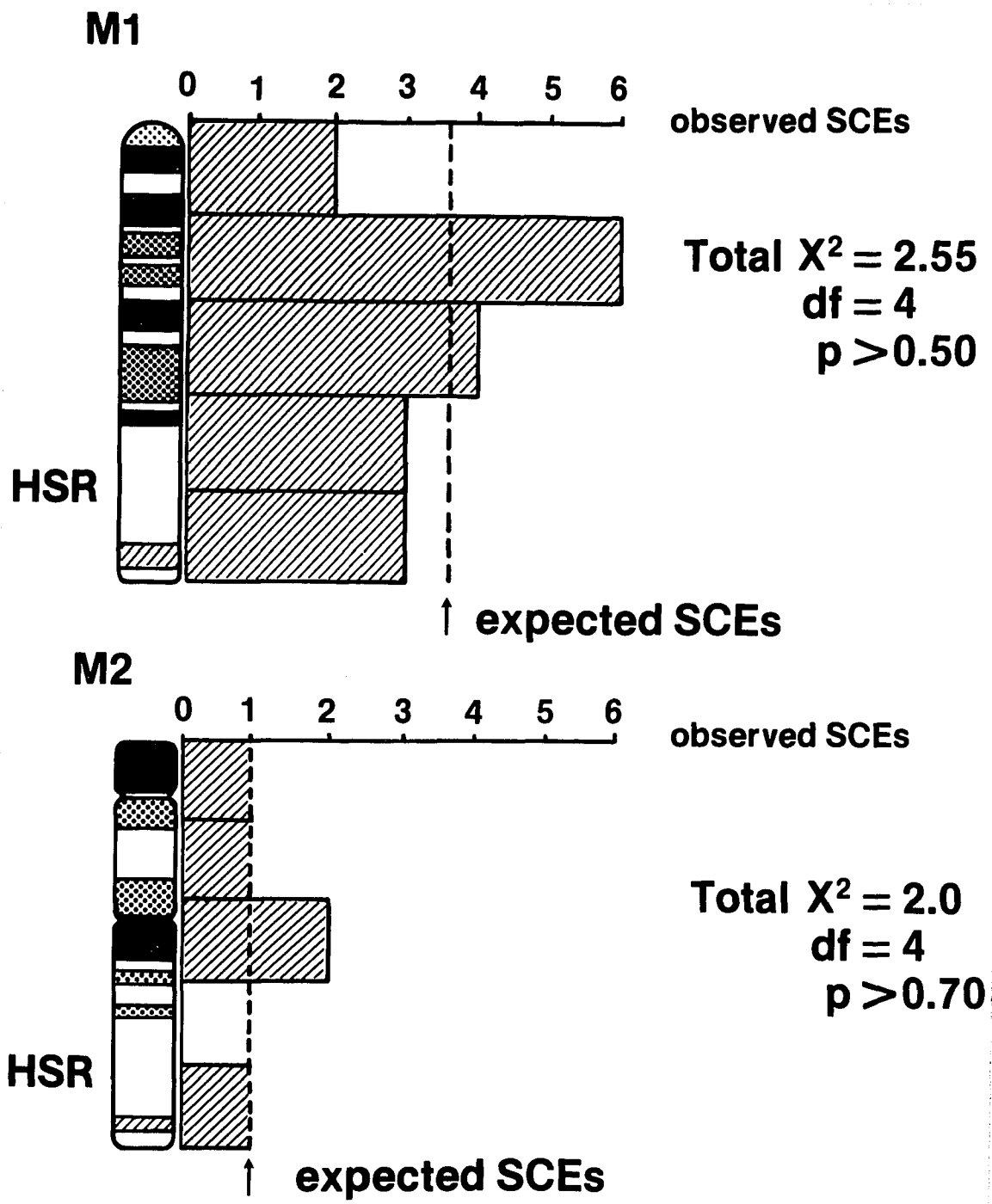

Figure 4. Chi-square analysis of SCE location in HSR-bearing markers.

were true, then the total frequency of SCE within HSR-bearing markers might differ from the expected levels based on other areas of the genome.

An analogous situation to HSR formation may exist in the generation and maintenance of heterochromatic regions. Numerous studies on the SCE frequency in heterochromatin have reported that the SCEs occur at lower frequencies within heterochromatic regions, but in increased frequencies at the junctions of euchromatin and heterochromatin (Carrano and Wolff 1975, Bostock and Christie 1976, Kato 1979, Crossen 1983). Assuming that unequal SCE may have a role in the evolution and the maintenance of multigene families (Smith 1973, Tartof 1974, Southern 1975, Smith 1976), increased frequencies of SCE at the edges of heterochromatic areas could be responsible for the generation or depletion of repeated sequences. Similarly, SCE might be limited to the edges of HSRs, increasing the possibility of unequal SCE and gene sequence amplification or deletion. By analogy, the suppression of SCE within HSRs might be expected to protect and maintain these gene sequences.

As previously mentioned, the distribution of SCE along both markers was not significantly different from random distribution. Therefore, it can be assumed that SCEs do not occur with either increased or decreased frequencies in any region of the HSR-bearing chromosomes. Thus, the results of this study do not support the unequal SCE theory of HSR formation.

Two other theories have been proposed to explain the mechanism of HSR formation: 1) the uptake and integration of DNA

from extrachromosomal sources (Wigler et al. 1979) or 2) saltatory or disproportionate replication (Schimke, 1981). The first theory hypothesizes that HSR-formation could arise because extracellular DNA could have been engulfed by cells, replicated, and integrated into a chromosome of another cell. However, Fournier and Ruddle (1977) have demonstrated that the chromosomal site of integration is not specific; hence, this does not appear to be a likely mechanism for HSR formation because numerous copies of extracellular DNA would have to integrate at the same site in a single cell. The likelyhood of this happening would be exceedingly small.

The second major theory of DNA amplification is saltatory or disproportionate replication. In this model, replication is initiated at the same origin of replication more than once within a given S phase of a cell cycle, allowing gene segments to be amplified quickly without numerous intervening mitoses (Schimke et al. 1981). Botchan et al. (1979) described this process as the "onion skin" configuration where DNA replication-elongation slows down and/or ceases at some site within the chromosome prior to joining the replication fork proceeding from the adjacent origin of replication. End to end ligation of the duplicated DNA strands and recombination would be needed to insert the linearly amplified region into the chromosome in the vicinity of the resident gene (Schimke et al. 1981). Schimke et al. (1981) also suggested that if DNA replicates from matrix-bound looped structures, then the free ends of DNA may close together, being circularized, thus forming a vehicle for the

rolling-circle replication method which could produce directly repeated DNA segments. A recombinational event would be required to insert the repeating gene segment into the chromosomes at the site of the resident gene, resulting in an HSR.

With the latter two gene amplification models (uptake of extra chromosomal DNA or saltatory or disproportionate replication) the frequency of SCE occurring in cells with HSRs would not be expected to differ from that occurring in non-HSR-bearing cells. Conversely, if unequal SCE were the mechanism of gene amplification and HSR formation, than elevated SCE frequencies would be expected in HSR-bearing chromosomes. The results of this thesis do not support the unequal SCE mechanism but are consistent with the theories of uptake of extrachromosomal DNA or saltatory or disproporationate replication of DNA segments as the mechanism of gene amplification and HSR formation in somatic cells.

SUMMARY AND CONCLUSIONS

The MTX ^{R} L5178YR cell line, including and excluding its HSR-bearing marker chromosomes, did not demonstrate a statistically significant difference in the frequency of SCE/U.L. when compared to its MTX ^{S} parental cell line ($p = 0.251$, $p = 0.116$). Additionally, the SCE frequency of the HSR-bearing markers also fell within the expected range ($p > 0.05$) and their distribution was random according to Poisson expectations ($p > 0.20$). Thus, in this cell line, HSRs do not appear to be either shielded from the occurrence of SCE or to be targeted areas of enhanced SCE. SCEs were also found to occur randomly along HSR-bearing markers (M1: $p > 0.50$, M2: $p > 0.70$).

These data do not support the model of unequal crossing over as a mechanism of gene amplification important in HSR formation. Instead, the data are consistent with either uptake of extrachromosomal DNA or the saltatory or disproportionate model of gene amplification. This research suggest that the understanding of the actual mechanism responsible for HSR formation in MTX ^{R} cell lines will require further study at the nucleotide, rather than chromsome level.

APPENDIX A

SUPPLEMENTARY DATA

ON SCE IN CELL LINES AND CHI-SQUARE

Table A-1. Observed SCE frequency in L5178YS cells.

Cell	Chromosome length	SCE/cell	SCE/U.L.
1	636.1	7	0.0110
2	606.5	16	0.0264
3	580.2	7	0.0121
4	442.8	6	0.0136
5	592.5	3	0.0051
6	492.2	10	0.0203
7	482.3	8	0.0166
8	458.2	10	0.0218
9	493.6	8	0.0162
10	568.6	4	0.0070
11	636.3	8	0.0126
12	398.2	9	0.0226
13	536.1	5	0.0093
14	511.6	6	0.0117
15	437.3	11	0.0252
16	498.7	7	0.0140
17	573.4	5	0.0087
18	561.3	9	0.0160
19	497.6	6	0.0121
20	498.2	8	0.0161
21	570.5	9	0.0158
22	528.0	7	0.0133
23	539.6	11	0.0204
24	319.8	4	0.0125
25	509.6	4	0.0078
26	392.2	6	0.0153
27	529.8	8	0.0151
28	463.4	5	0.0108
29	547.4	10	0.0183
30	473.6	4	0.0084
31	544.5	9	0.0165
32	411.9	6	0.0146
33	525.0	10	0.0190
34	525.4	6	0.0114
35	360.9	6	0.0166
36	525.6	6	0.0114
37	475.0	9	0.0189
38	499.0	6	0.0120
39	586.1	6	0.0102
40	570.6	14	0.0245
41	372.1	6	0.0161
42	476.1	6	0.0126

Table A-1. Continued

Cell	Chromosome length	SCE/cell	SCE/U.L.
43	471.9	17	0.0360
44	441.6	10	0.0226
45	504.1	9	0.0179
46	585.2	6	0.0103
47	489.2	6	0.0123
48	468.6	6	0.0128
49	466.0	7	0.0150
50	488.6	8	0.0164

Table A-2. Observed SCE frequency in L5178YR cells.

Cell	Chromosome length	SCE/cell	SCE/U.L.
1	519.3	6	0.0116
2	475.8	8	0.0168
3	521.6	15	0.0288
4	411.9	10	0.0243
5	615.7	4	0.0065
6	442.4	12	0.0271
7	413.9	8	0.0193
8	605.8	9	0.0149
9	615.2	8	0.0130
10	581.0	8	0.0138
11	550.5	11	0.0199
12	639.8	10	0.0156
13	560.3	7	0.0125
14	519.3	12	0.0231
15	397.1	15	0.0378
16	450.8	6	0.0133
17	539.2	15	0.0278
18	519.5	11	0.0212
19	594.7	6	0.0101
20	642.0	10	0.0156
21	608.1	8	0.0132
22	485.3	6	0.0124
23	554.8	7	0.0126
24	485.6	12	0.0247
25	541.5	10	0.0185
26	556.6	13	0.0234
27	577.3	8	0.0139
28	513.5	11	0.0214
29	466.0	9	0.0193
30	650.1	6	0.0092
31	522.6	8	0.0153
32	453.3	3	0.0066
33	619.1	11	0.0178
34	584.6	8	0.0137
35	529.0	8	0.0151
36	597.8	12	0.0201
37	579.3	11	0.0190
38	752.0	4	0.0053
39	540.1	8	0.0148
40	614.4	8	0.0130
41	808.3	8	0.0099
42	516.2	4	0.0077

Table A-2. Continued.

Cell	Chromosome length	SCE/cell	SCE/U.L.
43	583.3	10	0.0171
44	480.9	8	0.0166
45	446.9	6	0.0134
46	584.7	15	0.0257
47	587.2	6	0.0102
48	494.1	10	0.0202
49	609.0	6	0.0099
50	450.2	9	0.0200

Table A-3. Observed SCE frequency in L5178YR cells - (M1 + M2).

Cell	Chromosome length -(M1 + M2)	SCE/non-HSR-bearing markers	SCE/U.L.
1	446.1	6	0.0129
2	410.2	7	0.0171
3	470.6	15	0.0319
4	370.7	10	0.0270
5	554.5	4	0.0072
6	390.3	12	0.0307
7	370.4	8	0.0216
8	545.1	9	0.0165
9	546.1	8	0.0146
10	543.4	8	0.0147
11	516.7	11	0.0213
12	556.5	10	0.0180
13	529.6	7	0.0132
14	482.9	12	0.0248
15	371.0	15	0.0404
16	424.0	6	0.0142
17	509.6	15	0.0294
18	480.2	11	0.0229
19	534.6	4	0.0075
20	565.8	10	0.0177
21	509.0	7	0.0138
22	438.9	4	0.0091
23	502.3	5	0.0100
24	435.1	11	0.0253
25	505.9	9	0.0178
26	524.1	12	0.0229
27	512.9	6	0.0117
28	489.1	11	0.0225
29	438.2	9	0.0205
30	573.9	6	0.0105
31	445.6	8	0.0180
32	423.2	3	0.0071
33	581.0	11	0.0189
34	557.1	8	0.0144
35	505.0	8	0.0158
36	559.3	11	0.0197
37	549.8	11	0.0200
38	706.3	3	0.0042
39	513.8	8	0.0156
40	575.2	8	0.0139
41	758.4	7	0.0092
42	483.0	4	0.0083

Table A-3. Continued.

Cell	Chromosome length -(M1 + M2)	SCE/non-HSR-bearing markers	SCE/U.L.
43	549.6	9	0.0164
44	455.8	8	0.0176
45	413.5	4	0.0098
46	549.4	12	0.0218
47	549.4	6	0.0109
48	459.5	10	0.0218
49	547.9	6	0.0110
50	398.9	9	0.0226

Table A-4. Chi-square analysis of SCE frequency in HSR-bearing markers

Cell	Observed SCE	Expected SCE	Chi-square value
1	0	0.6842	0.6842
2	1	1.1200	0.0129
3	0	1.6250	1.6250
4	0	1.1100	1.1100
5	0	0.4416	0.4416
6	0	1.6030	1.6030
7	0	0.9404	0.9404
8	0	1.0020	1.0020
9	0	1.0120	1.0120
10	0	0.5530	0.5530
11	0	0.7192	0.7192
12	0	1.4970	1.4970
13	0	0.4056	0.4056
14	0	0.9046	0.9046
15	0	1.0520	1.0520
16	0	0.3803	0.3803
17	0	0.8695	0.8695
18	0	0.9007	0.9007
19	2	0.4493	5.3520
20	0	1.3470	1.3470
21	1	1.3630	0.0967
22	2	0.4225	5.8900
23	2	0.5225	4.1780
24	1	1.2770	0.0601
25	1	0.6337	0.2117
26	1	0.7438	0.0882
27	2	0.7525	2.0681
28	0	0.5487	0.5487
29	0	0.5701	0.5701
30	0	0.7973	0.7973
31	0	1.3820	1.3820
32	0	0.2134	0.2134
33	0	0.7205	0.7205
34	0	0.3960	0.3960
35	1	0.3331	1.3352
36	1	0.7587	0.0764
37	0	0.5912	0.5912
38	1	0.1940	3.3486
39	0	0.4094	0.4094
40	0	0.5457	0.5457
41	1	0.4599	0.6343
42	0	0.2756	0.2756

Table A-4. Continued.

Cell	Observed SCE	Expected SCE	Chi-square value
43	1	0.5838	0.2967
44	0	0.4403	0.4403
45	2	0.3238	8.6771
46	3	0.7754	6.3823
47	0	0.4120	0.4120
48	0	0.7539	0.7539
49	0	0.6696	0.6696
50	0	1.1570	1.1570

REFERENCES

Balaban, G. and F. Gilbert, Homogeneously staining regions in direct preparations from human neuroblastomas. Cancer Res. 42, 1838-1842 (1982)

Balaban-Malenbaum, G. and F. Gilbert, Double minute chromosomes and homogeneously staining regions in chromosomes of a human neuroblastoma cell line. Science 198, 739-741 (1977)

Balaban-Malenbaum, G. and F. Gilbert, Origin of double minute chromosomes in human neuroblastoma cells. Am. J. Hum. Genetics 29, 21A (1978)

Balaban-Malenbaum, G. and F. Gilbert, The proposed origin of double minutes from homogeneously staining regions (HSR)-marker chromosomes in human neuroblastoma hybrid cell lines. Cancer Gen. Cytogenetics 2, 339-348 (1980)

Barker, P.E., V.F. Lau, and T.C. Hsu, A heterochromatic HSR in the karyotype of a human breast cancer cell line. Cancer Gen. Cytogenetics 1, 311-319 (1980)

Biedler, J.L., M.B. Meyers, R.H.F. Peterson, and B.A. Spengler, Marker chromosome with a homogeneously staining region (HSR) in vincristine resistant cells. Proc. Am. Assoc. Cancer Res. 21, 292 (1980)

Biedler, J.L. and B.A. Spengler, Metaphase chromosome anomaly: Associated with drug resistance and cell-specific products. Science 191, 185-187 (1976)

Bostock, C.J. and S. Christie, Analysis of the frequency of sister chromatid exchange in different regions of chromosomes of the kangaroo rat (Dipodomys oridii). Chromosoma 56, 275-287 (1976)

Botchan, M., W. Topp, and J. Sambrook, Studies on SV40 excision from cellular chromosomes. Cold Spring Harbor Symp. Quant. Biol. 43, 709-719 (1979)

Carrano, A.V. and S. Wolff, Distribution of sister chromatid exchange in euchromatin and heterochromatin of the Indian muntjac. Chromosoma 53, 361-369 (1975)

Chasin, L.A. and L. Graf, N. Ellis, M. Landzberg, and G. Urlaub, Gene amplification in dihydrofolate reductase-deficient mutants. In Gene Amplification, R.T. Schimke, Ed., pp 161-165 (1982)

Crossen, P.E., DNA replication and sister chromatid exchange in 9qh+. Cytogenet. Cell Genet., in press (1983)

Dolnick, B.J., R.J. Berenson, J.R. Bertino, R.J. Kaufman, J.H. Nunberg, and R.T. Schimke, Correlations of dihydrofolate reductase elevation with gene amplification in a homogeneously staining chromosomal region in L5178Y cells. J. Cell Biology 83, 394-402 (1979)

Fournier, R.E.K. and F.H. Ruddle, Stable association of the human transgenome and host murine chromosomes demonstrated with trispecific microcell hybrids. Proc. Natl. Acad. Sci. U.S.A. 74, 3937-3941 (1977)

German, J., Biological role for chromatid exchange in mammalian somatic cellsμ In Gene Amplification, R.T. Schimke, Ed., pp 307-312 (1982)

George, D.L. and V.E. Powers, Sister chromatid exchange frequency and DNA replication pattern within a homogeneously staining region. Am. J. Hum. Genetics 32, 70A (1980)

Harper, M.E. and R.E. Kellems, Molecular basis of methotrexate resistance. Cancer Bull. 33, 43-48 (1981)

Ikushima, T. and S. Wolff, Sister chromatid exchanges induced by light flashes to 5-bromodeoxyuridine and 5-iododeoxyuridine substituted Chinese hamster chromosomes. Exp. Cell Res. 87, 15-19 (1974)

Kato, E. Perferential occurrence of sister chromatid exchanges at heterochromatin-euchromatin junctions in the wallaby and hamster chromosomes. Chromosoma 74, 307-316 (1979)

Latt, S.A., Microfluorometric detection of deoxyribonucleic acid replication in human metaphase chromosomes. Proc. Natl. Acad. Sci. U.S.A. 70, 3395-3399 (1973)

Latt, S.A., Localization of sister chromatid exchanges in human chromosomes. Science 185, 74-76 (1974).

Meselson, M. and F.W. Stahl, The replication of DNA in Escherichia coli. Proc. Natl. Acad. Sci. U.S.A., 44, 671-682 (1958)

Morgan, W.F. and P.E. Crossen, The frequency and distribution of sister chromatid exchanges in human chromosomes. Hum. Genet. 38, 271-278 (1977)

Perry, P. and S. Wolff, New Giesma method for the differential staining of sister chromatids. Nature 251, 156-158 (1974)

Prescott, D.M. and M.A. Bender, Autoradiographic study of chromatid distribution of labeled DNA in two types of mammalian cells in vitro. Exptl. Cell Res. 29, 430-442 (1963)

Quinn, L.A., G.E. Moore, R.T. Morgan, and L.K. Woods, Cell lines from human colon carcinoma with unusual cell products, double minutes, and homogeneous staining regions, Cancer Res. 39, 4914-4924 (1979)

Schimke, R.T., R.J. Kaufman, F.W. Alt, and R.E. Kellems, Gene amplification and drug resistance in cultured murine cells. Science 202, 1051-1055 (1978)

Schimke, R.T., P.C. Brown, R.J. Kaufman, M. McGrogan, and D.L. Slate, Chromosomal and extrachromosomal localization of amplified dihydrofolate reductase genes in cultured mammalian cells. Cold Spring Har. Symp. Quant. Biol. 45, 785-797 (1981)

Schimke, R.T., Summary, In Gene Amplification R.T. Schimke, Ed., pp 317-331 (1982)

Smith, G., Unequal cross-over and the evoluation of multigene families. Cold Spring Har. Symp. Quant. Biol. 38, 507-513 (1973)

Smith, G., Evolution of repeated DNA sequences by unequal cross-over. Science 191, 528-535 (1976)

Southern, E.M., Long range periodicities in mouse satellite DNA. J. Molec. Biol. 94, 51-69 (1975)

Tartof, K.D., Unequal mitotic sister chromatin exchange as the mechanism of ribosomal RNA gene amplification. Proc. Natl. Acad. Sci. U.S.A. 71, 1274-1276 (1974)

Taylor, J.H. Sister chromatid exchanges in tritium-labeled chromosomes Genetics 43, 515-529 (1958)

Taylor, J.H., P.S. Woods, and W.L. Hughes, The organization and duplication of chromosomes as revealed by autoradiographic studies using tritium-labeled thymidine. Proc. Natl. Acad. Sci. U.S.A. 43, 122-128 (1957)

Trent, J.M., Cytogenetic analysis of human cells cloned in agar. In Cloning of Human Tumor Stem Cells S.E. Salmon, Ed., pp. 165-177 (1980)

Trent, J.M., Chromosomal alterations in clonogenic human tumor cells resistant to cytostatic agents. In Gene Amplification, R.T. Schimke, Ed., pp 99-105 (1982)

Wigler, M., R. Sweet, G.K. Sim, B. Wold., A. Pellicer, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel, Transformation of mammalian cells with genes from prokaryotes and eukaryotes. Cell 16, 777-785 (1979)

Wolff, S., Sister chromatid exchange. Annual Review, Genetics 11, 183-201 (1979)