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High-resolution measurement of breaks in prematurely condensed chromosomes by differential staining

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Abstract. A relatively simple method has been developed to improve the resolution for measuring breaks produced in interphase chromosomes by X rays or other agents following the induction of premature chromosome condensation (PCC). Mitotic HeLa cells, which induce PCC when fused with interphase cells, were obtained from cultures grown for several generations in 5-bromodeoxyuridine (BrdU). These were fused to cells from low-passage confluent cultures of normal human fibroblasts and subsequently stained by a modified fluorescence-plus-Giemsa (FPG) technique. Following this protocol the prematurely condensed chromosomes stain intensely, whereas the mitotic chromosomes of the inducer cell(s), which are intermingled with them, stain very lightly. With this technique the interphase chromosomes and their fragments can be identified unequivocally, making scoring much easier and more accurate. The frequency of breaks produced in G1 phase AG1522 human fibroblasts immediately following X-ray doses of 58 and 117 rad was 3.68 and 7.38 per cell, respectively. Use of this technique should allow the detection of damage from ionizing radiation at doses lower than 10 rad.

Introduction

Factors in the cytoplasm of a mitotic cell can cause the chromatin of an interphase cell to condense prematurely when the two are fused together (Johnson and Rao 1970). The resulting prematurely condensed chromosomes (PCCs) are characteristic of the particular cell cycle phase at the time of fusion (i.e., G1, S, or G2) (Rao et al. 1977). This technique can be used to visualize damage to interphase chromosomes immediately after treatment with radiation or drugs. Waldren and Johnson (1974) showed that about ten times more chromosome breaks are detectable in the PCCs of X-irradiated G1 phase HeLa cells than in similar cells scored for chromosome damage at mitosis. At about the same time, Hittelman and Rao (1974) found that X-ray induced breaks in the chromosomes of G2 CHO cells are twice as numerous as in mitotic cells. More recently Hittelman and Pollard have demonstrated the rejoining of breaks in PCCs of irradiated G2 CHO cells (1982a) and of quiescent human fibroblasts exposed to neocarzinostatin (1982b). Also using the PCC technique, Johnson et al. (1982) have measured rejoining rates in X-irradiated G1 HeLa cells and embryonic chick erythrocytes.

Despite its potential power as a tool in the study of such phenomena, PCC analysis has been less than enthusiastically embraced for this purpose by all but a few cytogenetics laboratories. Perhaps the main reason is that it has a few, largely technical, drawbacks.

Like mitotic chromosomes, prematurely condensed G2 chromosomes are bipartite, so damage can be quantified by the usual cytogenetic criteria. Chromatid deletions and exchanges are relatively easy to score. Prematurely condensed G1 chromosomes, however, appear unipartite since they are condensed from chromatin that has not yet duplicated. To assess damage in G1 PCCs one must score the total number of prematurely condensed chromatin bodies. In untreated cells, this number is equal to the number of metaphase chromosomes, so a diploid cell receiving just one break will yield $2n+1$ pieces. Most PCC studies involving X-ray induced breakage have used cell lines that are, to some degree, aneuploid. For unirradiated HeLa cells the number of "G1 chromosomes" per cell ranges from 42–72 (Waldren and Johnson 1974); therefore, detecting only a few breaks per cell is not possible.

A more serious general problem for quantitative analysis of PCCs is that usually some or all of the prematurely condensed fragments are interspersed among the mitotic chromosomes of the inducer cell. Thus, PCCs are often obscured by overlapping mitotic chromosomes, and small acrocentric mitotic chromosomes are easily mistaken for PCCs (and vice versa). Furthermore, when mitotic inducer cells are collected, the mitotic index is never 100%. If this population is contaminated with even a few percent of interphase cells, then the probability of inadvertently scoring a PCC in the wrong fusion product becomes a problem. For high resolution measurements, therefore, it is extremely important to be able to recognize all PCCs and their fragments, and to distinguish PCCs resulting from undesirable fusions.

We have developed a technique, based on the differential Giemsa staining of BrdU-substituted chromosomes (Perry and Wolff 1974), that overcomes these problems and have tested it by measuring breaks produced by X rays.

Materials and methods

Cell-lines. Low-passage normal human fibroblast AG1522 cells were obtained from Dr. Arthur Greene, N.I.A. Cell Repository. These were grown in alpha-MEM (Gibco) supplemented with 15% fetal bovine serum. Under these condi-

tions the plating efficiency was 65%, and the cell population doubling time was about 17 h.

S3 HeLa cells were originally obtained from Dr. L.J. Tolmach, Washington University, St. Louis and were maintained in Ham's F12 medium (Gibco) supplemented with 8% fetal bovine serum and 5×10^{-6} M 5-bromodeoxyuridine (BrdU).

Cultures were kept at 37° C, in a humidified atmosphere of 5% CO_2 in air, in growth media containing penicillin and streptomycin. About 6 days before an experiment, 5×10^5 AG1522 cells were seeded into each of a number of 25-cm^2 T-flasks. Two days before a fusion, 2×10^6 HeLa cells, from which mitotic inducer cells would be obtained, were seeded into several 150-cm^2 T-flasks.

Cell collection and fusion. Approximately 24 h before cells were to be fused, hydroxyurea (HU) was added to a final concentration of 2.0 mM to all HeLa cultures. After 12 h the HU block was released, and cells were allowed to progress for 7 h in medium without HU. At this time loosely adhering cells were selectively detached (shaken-off) and discarded. Prewarmed medium was then re-added together with 0.1 μg/ml Colcemid (Gibco). After the synchronized HeLa cells had reached mitosis about 5 h later, they were collected by selective detachment, and the pooled suspension was centrifuged. The mitotic HeLa cells were then resuspended in Hanks' balanced salts solution made up without the addition of glucose (HBSS-G), and containing 0.2 μg/ml Colcemid. These cells were centrifuged again, resuspended in HBSS-G plus Colcemid, and counted using a Particle Data electronic cell counter. A small sample was then removed for determination of the mitotic index, and the remainder of the BrdU-containing mitotic HeLa cells were stored on ice until fusion. Usually more than 95% of the HeLa cells collected in this manner were in mitotis.

Following a procedure similar to the one described by Hittelman and Rao (1974), density-inhibited monolayers of AG1522 human fibroblasts were removed from 25-cm^2 T-flasks by treatment with 0.03% trypsin (Difco) in saline D1 (Ham and Puck 1962), washed twice in HBSS-G plus 0.2 μg/ml Colcemid, and resuspended in the same salt solution before placing on ice.

For each fusion approximately 7.5×10^5 mitotic HeLa cells were mixed with 7.5×10^5 interphase AG1522 cells in a 15-ml conical plastic centrifuge test tube. After centrifugation the cell pellet was resuspended in 0.5 ml HBSS-G with Colcemid, and the tubes were placed in an ice bath. UV-inactivated Sendai virus was added to give a final concentration of 150 hemagglutinating units/ml. Tubes were immediately mixed, and returned to the ice bath for a total of 15 min. Irradiations were done at this point, keeping the clumped (but not fused) cell suspensions at 0° C to prevent the rejoining of fragments. A GE Maxitron 300 X-ray unit operating at 280 kV and with 1.27 mm Al filtration (H.V.L. = 0.3 mm Cu) was used to deliver the doses at a rate of 59 rad/min. Dose rates were measured using an EXRADIN 0.05 cm^3 tissue equivalent ionization chamber. After irradiation the cells were centrifuged gently to form a loose pellet, and tubes were then placed into a 37° C waterbath to initiate cell fusion. After 10 min, 5 ml of growth media (with serum and 0.2 μg/ml Colcemid) was added. The tubes were incubated for an additional 35 min, then centrifuged briefly and the supernatant removed. The cell pellet was gently resuspended in 8 ml prewarmed KCl (0.075 M), and the tubes were allowed to stand at room temperature for 15 min. After the hypotonic treatment, 3 ml methanol:acetic acid (3:1) was added all at once to each tube, without removing the KCl. The suspension was centrifuged and all but 0.5 ml of the supernatant was discarded. The cells were resuspended in this small volume, and after 1 ml methanol-acetic acid fixative had been added dropwise, an additional 3 ml was added. The tubes were then allowed to stand at room temperature for at least 10 min. Cell suspensions were washed twice in fresh fixative and resuspended in a small volume before being dropped onto chilled, wet slides.

Procedure for differential staining. The staining technique involved a slight modification of the fluorescence-plus-Giemsa (FPG) method described by Perry and Wolff (1974). Slides were stained for 15 min in Sorensen's buffer (pH 6.8) containing 5 μg/ml Hoechst 33258, rinsed briefly with water and dried. Slides were mounted in buffer, using a number 1 coverslip, and ringed with rubber cement to prevent evaporation. The wet mounts were placed 6 cm from a rack of long-wave UV bulbs (Westinghouse FS20) for 8 h. Coverslips were then removed, the slides rinsed with water and placed into Coplin jars containing 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate) that had been preheated to 56° C. After 90 min, slides were gently, but thoroughly, rinsed with distilled water before staining in Giemsa (5% Gurr's R66 in Sorensen's buffer, pH 6.8) for 5–10 min.

Results

Figure 1 A shows the PCCs of a G1 phase AG1522 cell stained in the conventional way with Giemsa to illustrate the difficulty in scoring fragments intermingled with mitotic HeLa chromosomes. Figure 1 B shows the G1 chromosomes of a similar cell in which PCCs were induced by a BrdU-substituted mitotic HeLa cell, but where the slide was stained by a modified FPG method. Since the HeLa cells were grown for many generations in BrdU, their DNA was bifilarly substituted with this thymidine analogue. Subsequent treatment with Hoechst 33258 dye followed by exposure to UV light caused a very faint staining with Giemsa. The staining properties of the AG1522 chromosomes, which were never exposed to BrdU, were not affected by Hoechst-plus-light treatment and consequently stained very intensely.

The centromeric regions of some PCCs are achromatic, and this causes perhaps the largest uncertainty in the scoring of fragments. The number of chromosomes per cell scored for mitotic and G1 cells (after premature condensation) is shown in Figure 2. Although the spread about the modal number is greater for PCCs, the average number of AG1522 PCCs per cell is virtually identical to the average number of metaphase chromosomes per cell. While less dispersion in the number of G1 PCCs would be desirable, the spread remains small enough to allow a relatively high resolution measurement of the breaks produced by agents such as X rays.

Incorrect fusion products (the occasional fusion between a contaminating G1 HeLa cell and a mitotic HeLa cell) were readily identified since all HeLa chromosomes stained lightly.

More than 25% of the G1 AG1522 cells in the fusion mixture actually took part in a fusion event with one or

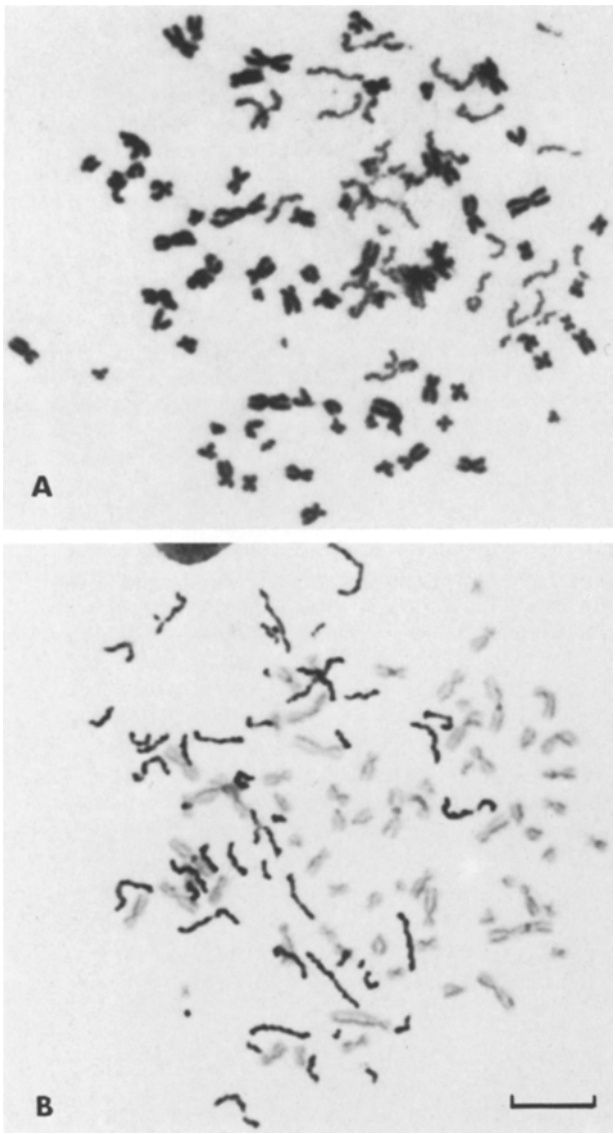

Fig. 1. Prematurely condensed chromosomes (PCCs) of G1 phase AG1522 normal human fibroblasts. The mitotic chromosomes in each spread are from S3 HeLa cells. **A** Conventional Giemsa preparation of an unirradiated cell, illustrating the difficulty of scoring PCCs. **B** Differential staining of a similar cell that had received 58 rad of 280 kVp X rays. Note the relative ease and accuracy with which PCCs can be counted among the faintly stained BrdU-containing chromosomes. The cell was scored as containing a total of 50 chromosomes and fragments indicating that four breaks had probably resulted from this treatment. Bar represents 10 μm

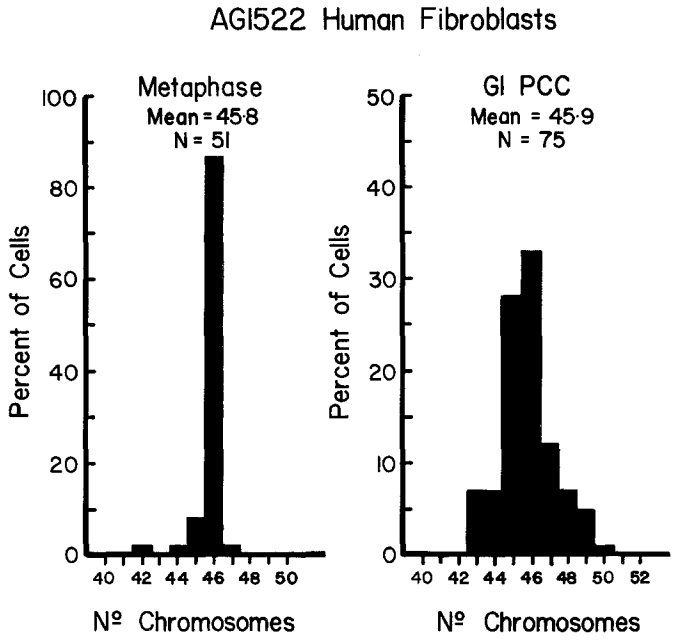

Fig. 2. The number of chromosomes per AG1522 cell scored at metaphase (*left*) and the number of prematurely condensed chromosomes (PCCs) per density-inhibited (G1) AG1522 cell (*right*). Note that the means for the two distributions are virtually identical.

Table 1. Induction of breaks by 280 kVp X rays in prematurely condensed chromosomes (PCCs) of plateau phase AG1522 human fibroblasts

Dose (rad)	No. cells analyzed	PCCs + fragments per cell (mean ± S.E.)	Net fragments (breaks per cell)	Breaks/ cell/rad
0	50	45.8 + 0.22	0.00	–
58	62	49.4 + 0.46	3.68	0.063
117	86	53.1 + 0.37	7.38	0.063

more mitotic HeLa cells. Premature chromosome condensation was successfully induced in virtually all AG1522 cells that did fuse with mitotic HeLa cells. Since neither the fusion frequency nor the PCC frequency differed for the two doses tested, it is reasonable to conclude that the measurements of chromosome breakage are representative of the damage that occurs in the irradiated cell population as a whole.

Table 1 shows the number of fragments (breaks) produced immediately after plateau phase AG1522 cells were irradiated to total doses of 58 or 117 per rad. The data show that the yield of breaks/per rad is quite high and that PCC fragments are produced in a dose-dependent manner. If the dose-response to X rays is indeed linear, as the data suggest, then we would expect an average of about 20 breaks per cell after a dose of 330 rad. In another experiment, which will be reported elsewhere in more detail, the average frequency of chromosomal aberrations after identical cultures had been given 330 rad, subcultured, and allowed to progress to mitosis was 1.04 per cell. These observations confirm previous work (Waldren and Johnson 1974) that the number of breaks measured immediately after irradiation in prematurely condensed G1 chromosomes is at least an order of magnitude greater than that seen after cells reach mitosis.

Discussion

We have developed a system that should overcome some of the difficulties previously associated with the assessment of damage in PCCs. The large increase in statistical resolution should allow the inherent sensitivity of the PCC technique to be utilized more effectively. Several studies that previously were impractical (if not impossible) might now be attempted. For example, statistical calculations using a Monte Carlo computer method to simulate the distribution of radiation-induced PCC fragmentation indicate that, with the present system, we should easily be able to measure

X-ray induced chromosome damage down to 5 rad by scoring as few as 200–300 cells. This, of course, assumes that the break frequency per rad remains at least as high as we have presently reported. We are currently working to determine a dose-response relationship for G1 chromosome breakage following ionizing radiation in this low dose range. Preliminary results also indicate that this system is well-suited for experiments involving the rejoining of G1 chromosome breaks, especially since confluent cultures of AG1522 cells are "contact inhibited" and would not move through the cell cycle during prolonged incubation periods while rejoining occurs. Differential staining of mitotic inducer cells would also aid studies involving PCCs for cytokinetic measurements. Here contaminating interphase cells from the "mitotic" inducer population can form PCCs that are indistinguishable from those induced by fusion with the test population. Differential staining would allow rapid identification of these undesirable fusion products.

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