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Ionizing Radiation Induces Frequent Translocations with Delayed Replication and Condensation

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ABSTRACT

Certain chromosome rearrangements display a significant delay in replication timing that is associated with a delay in mitotic chromosome condensation. Chromosomes with delay in replication timing/delay in mitotic chromosome condensation participate in frequent secondary rearrangements, indicating that cells with delay in replication timing/delay in mitotic chromosome condensation display chromosomal instability. In this report, we show that exposing cell lines or primary blood lymphocytes to ionizing radiation results in chromosomes with the delay in replication timing/delay in mitotic chromosome condensation phenotype, and that the delay in replication timing/delay in mitotic chromosome condensation phenotype occurs predominantly on chromosome translocations. In addition, exposing mice to ionizing radiation also induces cells with delay in replication timing/delay in mitotic chromosome condensation chromosomes that persist for as long as 2 years. Cells containing delay in replication timing/delay in mitotic chromosome condensation chromosomes frequently display hyperdiploid karyotypes, indicating that delay in replication timing/delay in mitotic chromosome condensation is associated with aneuploidy. Finally, using a chromosome engineering strategy, we show that only a subset of chromosome translocations displays delay in replication timing/delay in mitotic chromosome condensation. Our results indicate that specific chromosome rearrangements result in the generation of the delay in replication timing/delay in mitotic chromosome condensation phenotype and that this phenotype occurs frequently in cells exposed to ionizing radiation both *in vitro* and *in vivo*.

INTRODUCTION

Genome wide, chromosomal rearrangements are one of the most common cytogenetic findings associated with malignant solid tumors. Recent surveys described >2,000 recurrent chromosomal aberrations occurring in many different types of cancers (1). The relationship between oncogenesis and the majority of these recurrent chromosomal aberrations is currently unknown. We identified previously an abnormal phenotype associated with a subset of tumor-derived chromosome rearrangements (2). We found that four different chromosome rearrangements displayed a significant delay in the replication timing of the entire chromosome. This delay in replication timing phenotype is characterized by a 2- to 3-hour delay in both the initiation as well as the completion of DNA synthesis along the entire length of the chromosome, whereas the other chromosomes within the same cell show normal patterns of replication. Chromosomes with the delay in replication timing phenotype also display a significant delay in mitotic chromosome condensation. The delay in mitotic chromosome condensation phenotype is characterized by an under-condensed appearance that is associated with a lack of the mitosis-specific phosphorylation of histone H3 on serine 10. Chromosomes with delay in mitotic chromosome condensation were present in 5 of 7 tumor cell lines and 5 of 13 primary tumor samples, suggesting that chromosomes with delay in mitotic chromosome condensation are common in tumor cells *in vitro* and *in vivo*. Importantly, delay in replication timing/delay in mitotic chromosome condensation does not occur on nonrearranged chromosomes present in tumor cell lines, in primary skin fibroblasts, or in primary blood lymphocytes, suggesting that the delay in replication timing/delay in mitotic chromosome condensation phenotype is induced by chromosome rearrangement. Furthermore, chromosomes with delay in replication timing/delay in mitotic chromosome condensation are unstable and participate in frequent secondary rearrangements and translocations, and, therefore, cells containing delay in replication timing/delay in mitotic chromosome condensation display a chromosomal instability (2).

One limitation in the analysis of tumor-derived chromosomes is that the event(s) that initiated the delay in replication timing/delay in mitotic chromosome condensation phenotype could not be determined, especially given the propensity of these chromosomes to undergo additional rearrangements (2). Therefore, it is currently not known whether the delay in replication timing/delay in mitotic chromosome condensation phenotype is induced by an epigenetic modification that occurs on damaged chromosomes or due to a genetic modification that occurred on these rearranged chromosomes. Furthermore, it is not known which types of chromosomal alterations, *e.g.,* translocations, deletions, inversions, or insertions, or how frequently these alterations can give rise to the delay in replication timing/delay in mitotic chromosome condensation phenotype. It is also not known whether or not delay in replication timing/delay in mitotic chromosome condensation is restricted to tumor cells or whether it can arise in normal cells either *in vitro* or *in vivo*. In this report we show that delay in replication timing/delay in mitotic chromosome condensation occurs frequently after exposure of cell lines or primary cells to ionizing radiation *in vitro* or *in vivo*. We also show that a subset of chromosome translocations induced by Cre/*lox*P mediated "chromosome engineering" result in this delayed phenotype. These observations indicate that delay in replication timing/delay in mitotic chromosome condensation occurs only on specific chromosome rearrangements, indicating that delay in replication timing/delay in mitotic chromosome condensation is controlled by a *cis*-acting mechanism.

MATERIALS AND METHODS

Cell Culture. GM639 cells are an SV40 T-antigen–transformed human fibroblast cell line (American Type Culture Collection, Manassas, VA), and HTD114 cells are a human APRT null cell line (3). APRT selection included 10 μg/mL azaserine (Sigma, St. Louis, MO) and 10 μg/mL adenine (Sigma). These two cell lines were grown in DMEM supplemented with 10% bovine calf serum (Hyclone, Logan, UT). Primary blood lymphocytes were isolated after venipuncture into a Vacutainer CPT (Becton Dickinson, Franklin Lakes, NJ) per the manufacturer's recommendations and grown in 5 mL RPMI 1640 (Life Technologies, Inc., Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone) and 1% phytohemagglutinin (Life Technologies, Inc.). All of the cells were grown in a humidified incubator at 37°C in a 5% carbon dioxide atmosphere.

Ionizing Irradiation. GM639 or HTD114 cells were trypsinized and transferred to polypropylene tubes and exposed at room temperature to a ^{137}Cs-

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gamma radiation source (147 rad/min) to achieve the indicated dosages. Irradiated cells were transferred to tissue culture dishes and allowed to recover in fresh media for the indicated times before mitotic harvests. Twenty-four hours after isolating primary blood lymphocytes, they were irradiated as above and then allowed to recover for up to 3 more days before mitotic harvest.

Mitotic Harvests. In the absence of Colcemid, trypsinized cells or primary blood lymphocytes were centrifuged at 1,000 rpm for 10 minutes in a swinging bucket rotor. The cell pellet was resuspended in 75 mmol/L potassium chloride for 15 minutes at 37°C, recentrifuged at 1,000 rpm for 10 minutes, and fixed in 3:1 methanol to acetic acid. Fixed cells were added drop-wise to microscope slides to make metaphase spreads using standard methods (4). To quantify the frequency of delay in mitotic chromosome condensation in different cell populations, we classified a chromosome as having delay in mitotic chromosome condensation if it displayed at least two of the following characteristics: (1) at least twice as long as any other chromosome within the same spread, (2) less than half as wide as any other chromosome within the same spread, and/or (3) contained a bend of >180° (2).

Replication Timing and Immunofluorescence. The bromodeoxyuridine (BrdUrd) replication timing assay was performed as described previously (2). Three days after irradiation and 2, 3, 4, 5, 6, 7, and 8 hours before mitotic harvest, asynchronously growing GM639 or primary blood lymphocytes were exposed to a pulse of 20 mg/mL of BrdUrd (Sigma) for 15 minutes, washed with PBS, and chased in medium containing 0.2 mmol/L thymidine. Mitotic cells were harvested in the absence of Colcemid. The cells were treated with 75 mmol/L KCl for 15 minutes at 37°C, fixed in 3:1 methanol to acetic acid, and dropped on wet slides. The chromosomes were denatured in 70% formamide in 2× SSC (1× SSC is 150 mmol/L NaCl and 15 mmol/L sodium citrate) at 70°C for 3 minutes. Incorporated BrdUrd was detected using a FITC-labeled anti-BrdUrd antibody (Becton Dickinson). Slides were stained with propidium iodide (0.3 mg/mL), coverslipped, and viewed under UV fluorescence (Zeiss, Anaheim, CA).

Fluorescence *In situ* Hybridization (FISH). Whole chromosome paints were used according to the manufacturer's recommendations (American Laboratory Technologies, Arlington, VA, and Vysis, Downers Grove, IL). DNA probes were nick-translated using standard protocols to incorporate digoxigenin-dUTP. Hybridizations were carried out on slides at 37°C for 16 hours. Final probe concentrations varied from 40 to 60 ng/μL and were detected as described (5). Detection of digoxigenin-dUTP probes used a three-step incubation of slides with sheep FITC-conjugated antidigoxigenin antibodies (Roche, Basel, Switzerland) followed by rabbit FITC-conjugated antisheep antibodies (Roche) followed by goat FITC-conjugated antirabbit antibodies (The Jackson Laboratory, Bar Harbor, MN). Slides were stained with 4′, 6-diamidino-2-phenylindole (12.5 μg/mL) or propidium iodide (0.3 μg/mL), coverslipped, and viewed under UV fluorescence with FITC filters (Zeiss).

***In vivo* Analysis of Delay in Mitotic Chromosome Condensation.** Hybrid mice (B6D2F1) were exposed to 7.5 Gy of ^{137}Cs-gamma radiation only on their right sides, as described previously (6). After lag times ranging from 3 days to 23 months, the mice were sacrificed and primary cultures established from enzymatically digested ear and kidney tissues, as described (6), except the total cellular contents of each organ were plated into two T75 tissue culture flasks. The primary cultures were grown in DMEM supplemented with 15% fetal bovine serum for ~5 days at which time they were treated with 30 ng/mL Colcemid for 12 hours and then harvested for cytogenetic analysis using standard protocols (4).

Chromosome Engineering. Three micrograms of the 5′AP-Neo plasmid were linearized at a unique *Not*I site, electroporated (300 volts, 950 μF in PBS; Bio-Rad, Hercules, CA) into HTD114 cells, and grown under 500 μg/mL Geneticin (Life Technologies, Inc.) selection for 10 to 14 days. Neomycin-resistant colonies were then pooled together. Three micrograms of the Hyg-3′RT plasmid was linearized at a unique *Not*I site, electroporated (300 volts, 950 μF in PBS; Bio-Rad) into the pooled Neomycin-resistant cells, and grown under 500 μg/mL Geneticin and 200 μg/mL Hygromycin selection for 10 to 14 days. Approximately 54 individual Neomycin-resistant, Hygromycin-resistant colonies ("parental clones") were picked and expanded into 6-cm tissue culture dishes, and ~2×10^6 cells were transiently cotransfected with 12 μg of Lipofectamine (Invitrogen, Carlsbad, CA), 1 μg of a green fluorescent protein expression plasmid (pCSGFP), and either 3 μg of a Cre recombinase expression plasmid (pBS185, Life Technologies, Inc.) or an empty vector control (pBluescript SK, Stratagene, La Jolla, CA). Average transfection efficiencies, based on the number of green fluorescent protein$^+$ cells, were between 5% and 10%. Cre-transfected cells were grown for 14 to 21 days in 10 μg/mL azaserine and 10 μg/mL adenine to select for colonies that have reconstituted the *Aprt* gene via a *lox*P mediated site-specific chromosomal translocation event ("R-lines").

RESULTS

Ionizing Radiation Induces the Delay in Replication Timing/Delay in Mitotic Chromosome Condensation Phenotype. Exposing mammalian cells to ionizing radiation is an efficient way of inducing chromosome rearrangements (reviewed in refs. 7–9). Therefore, to determine whether ionizing radiation can generate chromosomes with delay in replication timing/delay in mitotic chromosome condensation, we exposed two cell lines to increasing doses of ionizing radiation and assayed mitotic spreads for the presence of the delay in mitotic chromosome condensation phenotype. One poorly understood aspect of the delay in mitotic chromosome condensation phenotype is the extreme variability in the extent of mitotic chromosome condensation. Therefore, to quantify the frequency of delay in mitotic chromosome condensation in different cell populations we have classified a chromosome as having delay in mitotic chromosome condensation if it displays at least two of the following characteristics: (1) at least twice as long as any other chromosome within the same metaphase spread, (2) less than half as wide as any other chromosome within the same metaphase spread, and/or (3) contained a bend of >180° (2). In addition, because Colcemid treatment before mitotic harvest interferes with our ability to detect the delay in mitotic chromosome condensation phenotype (2), the studies described here were carried out in the absence of a Colcemid pretreatment step, unless otherwise indicated.

Exponentially growing cultures were exposed to increasing doses of ionizing radiation, allowed to recover for 1 to 24 days, and scored for the presence of delay in mitotic chromosome condensation. The frequency of chromosomes with delay in mitotic chromosome condensation increased with increasing dose in both GM639 and HTD114 cell lines, and the frequency of delay in mitotic chromosome condensation was maximal 3 to 7 days after exposure (Fig. 1, *A* and *B*). This analysis indicated that chromosomes with delay in mitotic chromosome condensation could be detected in >25% of the cells exposed to ionizing radiation and that chromosomes with delay in mitotic chromosome condensation can persist in the irradiated cells for at least 24 days in culture. Representative mitotic spreads containing chromosomes with delay in mitotic chromosome condensation are shown (Fig. 1, *C* and *D*). These observations indicate that chromosomes with delay in mitotic chromosome condensation occur frequently and can persist for many generations after a sublethal dose of ionizing radiation. Interestingly, we did not detect an increase in the frequency of delay in mitotic chromosome condensation at the 24-hour time point (Fig. 1*A*), suggesting that the delay in mitotic chromosome condensation phenotype is not expressed during the first mitosis after irradiation.

To determine whether the chromosomes with delay in mitotic chromosome condensation generated by ionizing radiation also display the delay in replication timing phenotype, we examined the replication timing of the delay in mitotic chromosome condensation chromosomes using a BrdUrd incorporation assay (Fig. 1*E*). Analyzing mitotic spreads harvested for early replication indicated that the fully condensed chromosomes incorporated BrdUrd along their length, whereas chromosomes with delay in mitotic chromosome condensation showed no BrdUrd incorporation (data not shown). Analysis of mitotic spreads harvested for late replication indicated that chromosomes with delay in mitotic chromosome condensation

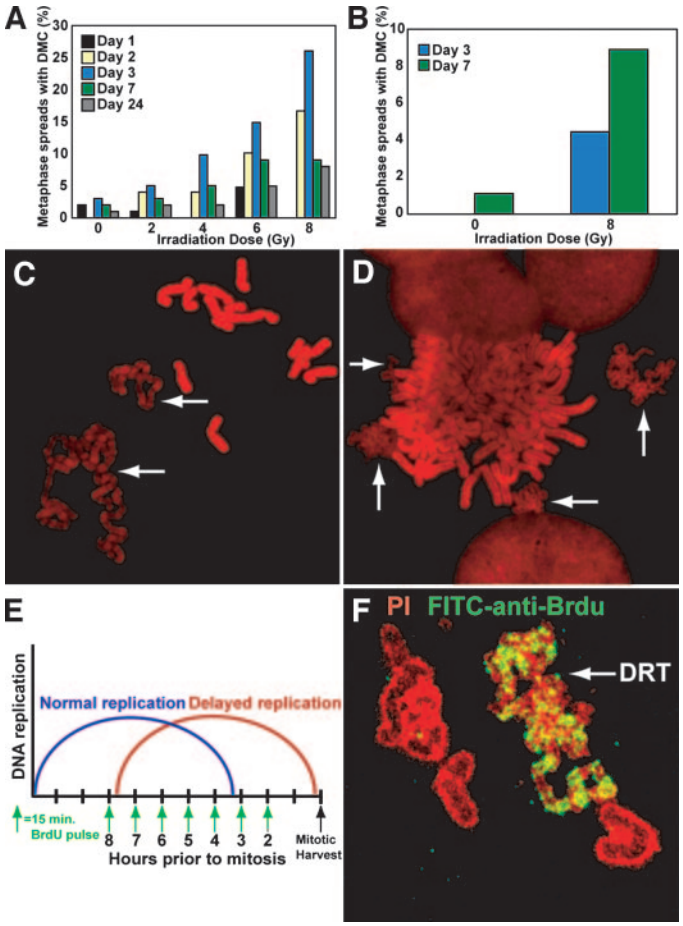

Fig. 1. Ionizing radiation induces DRT and DMC in immortalized cell lines. The frequency of DMC in metaphase spreads after different doses of ionizing radiation for GM639 (*A*) and HTD114 (*B*) cells. Mitotic spreads were harvested after 1, 2, 3, 7, and 24 days after 0, 2, 4, 6, or 8 Gy of ionizing radiation. A minimum of 100 mitotic spreads were scored for each time point and dose. Representative photomicrographs of DMC in metaphase spreads harvested from GM639 (*C*) and HTD114 (*D*) cells are shown. *E*, schematic diagram of BrdUrd replication timing assay. Chromosomes with DRT initiate and complete replication 2 to 3 hours after the normal chromosomes within the same cell. *F*, photomicrograph of an irradiated GM639 metaphase harvested for late replication (BrdUrd pulse 4 hours before mitotic harvest). Only the DMC chromosome is BrdUrd (BrdU) positive and therefore displays DRT (*arrow*). Chromosomes were counterstained with PI. (DRT, delay in replication timing; DMC, delay in mitotic chromosome condensation; PI, propidium iodide)

incorporated BrdUrd along their length at a time when the fully condensed chromosomes showed no BrdUrd incorporation (Fig. 1*F*), indicating that these delay in mitotic chromosome condensation chromosomes are late replicating and, therefore, display delay in replication timing as well as delay in mitotic chromosome condensation.

Ionizing Radiation Induces Delay in Replication Timing/Delay in Mitotic Chromosome Condensation in Primary Cultures of Human Blood Lymphocytes. To determine whether delay in replication timing/delay in mitotic chromosome condensation can be generated in primary cells, we exposed primary cultures of human blood lymphocytes to ionizing radiation and assayed for the presence of delay in mitotic chromosome condensation in mitotic spreads 3 days after irradiation. Similar to the cell lines, ionizing radiation induced delay in mitotic chromosome condensation in a significant fraction of primary blood lymphocytes in a dose-dependent manner (Fig. 2*A*). Examples of mitotic spreads from primary blood lymphocytes with delay in mitotic chromosome condensation are shown (Fig. 2, *B* and *C*). In addition, BrdUrd replication timing studies indicated that the chromosomes with delay in mitotic chromosome condensation in the irradiated primary blood lymphocytes also displayed delay in replication timing (Fig. 2*D*). These observations indicate that the delay in replication timing/delay in mitotic chromosome condensation phenotype can be detected in as many as 5% of primary blood lymphocytes exposed to 4 Gy of ionizing radiation.

Previous studies using human primary blood lymphocytes exposed to different types of ionizing radiation indicated that chromosome translocations were generated at relatively high frequencies. For example, 4 Gy of ^{60}Co gamma irradiation generated on average one translocation per cell (9). Therefore, to determine whether the delay in mitotic chromosome condensation chromosomes in our irradiated primary blood lymphocytes represented interchromosomal translocations and to estimate the frequency of interchromosomal translocations in these irradiated populations, we analyzed mitotic spreads using FISH with five different whole chromosome painting probes. Note that this analysis does not discriminate between simple and complex translocations, nor does it detect intrachromosomal rearrangements, which are also quite common after exposure to ionizing radiation (10). We anticipated that the frequency that any given chromosome would display the delay in mitotic chromosome condensation phenotype would be proportional to its size. Therefore, we chose whole chromosome paints representing five large chromosomes (HSA 1, 2, 3, 4, and 6). From a total of 1,178 metaphase spreads, we detected 7 mitotic spreads with delay in mitotic chromosome condensation chromosomes that hybridized to one of the probes. Importantly, 5 of the 7 cells with FISH-positive delay in mitotic chromosome condensation chromosomes also contained interchromosomal translocations involving that chromosome. For example, we detected two delay in mitotic chromosome condensation chromosomes within the same spread that hybridized, but only partially, to the chromosome 2-specific probe, indicating that these two chromosomes were inter-

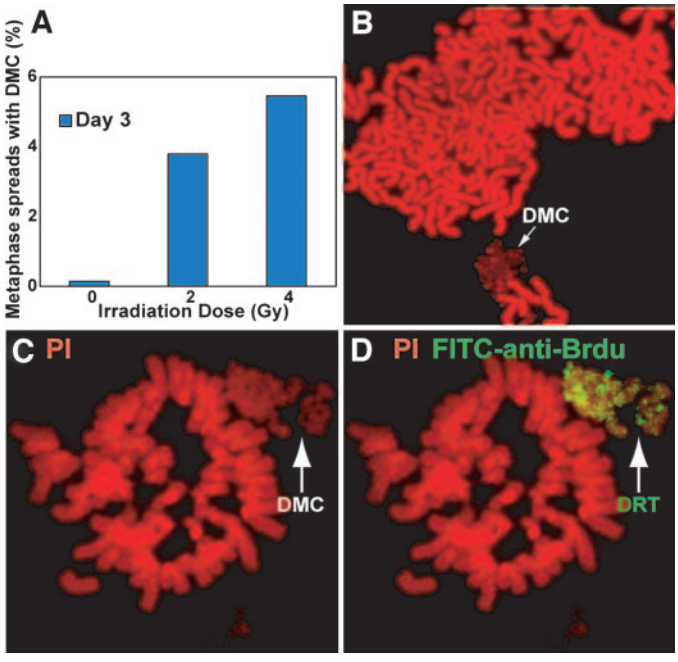

Fig. 2. Ionizing radiation causes DRT/DMC in primary blood lymphocytes. *A*, frequency of metaphase spreads with DMC in primary blood lymphocytes after ionizing radiation. Metaphase spreads were scored 3 days after exposure to 2 or 4 Gy of ionizing radiation. DMC was observed in 0.14% (1 of 708), 3.8% (11 of 279), and 5.5% (87 of 1,595) after 0, 2, and 4 Gy of ionizing radiation, respectively. *B*, photomicrograph of a representative hyperdiploid primary blood lymphocyte metaphase spread with DMC. *C* and *D*, photomicrograph of an irradiated primary blood lymphocyte metaphase harvested for late replication (BrdUrd pulse 4 hours before mitotic harvest). Only the DMC chromosome is BrdUrd (BrdU) positive and therefore displays DRT (*arrow*). The DNA was counterstained with PI. (DRT, delay in replication timing; DMC, delay in mitotic chromosome condensation; PI, propidium iodide)

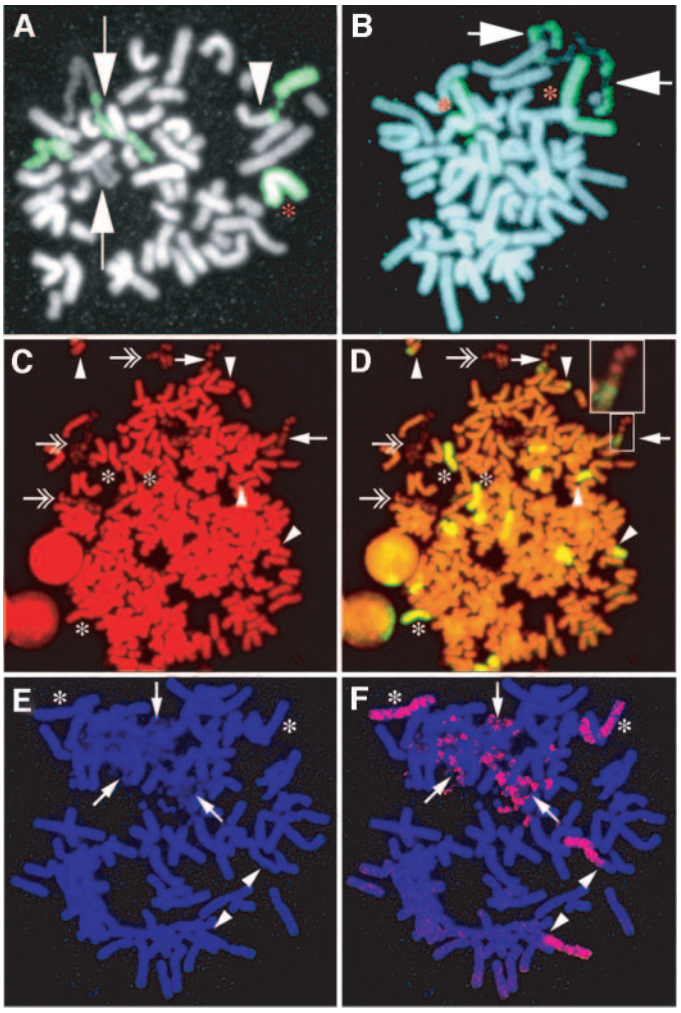

Fig. 3. Delay in mitotic chromosome condensation occurs on newly formed translocations. All panels represent metaphase spreads from primary blood lymphocytes scored 3 days after exposure to 4 Gy of ionizing radiation. Chromosomes hybridized to chromosome 2- (*A*, *B*, *F*, *green* or *red*) or chromosome 3-specific (*D*, *green*) whole chromosome paints. *A* and *B*, diploid metaphase spreads showing delay in mitotic chromosome condensation on chromosomes that hybridize with the chromosome 2 whole chromosome paints (*arrows*), partial hybridization indicates translocations. *A*, hybridization to a fully condensed chromosome 2 translocation (*arrowhead*) and nonrearranged chromosome 2s (*) were also detected. *C* and *D*, hyperdiploid metaphase spread showing numerous delays in mitotic chromosome condensation chromosomes. Two delay in mitotic chromosome condensation chromosomes partially hybridized to the chromosome 3 whole chromosome paints (*single arrows* and *inset box*) indicating translocations. Some delay in mitotic chromosome condensation chromosomes are whole chromosome paint negative (*double arrows*). Multiple, normally condensed chromosome 3 translocations are present (*arrowheads*). Several nonrearranged chromosome 3s are also present (*). *E* and *F*, hyperdiploid metaphase spread with an extremely under-condensed delay in mitotic chromosome condensation chromosome that hybridized to the chromosome 2 whole chromosome paint (*arrows*). Two fully condensed chromosome 2 translocations (*arrowheads*) and two normal chromosome 2s (*) are also present. The DNA was stained with either 4′, 6-diamidino-2-phenylindole (*A*, *B*, *E*, and *F*) or propidium iodide (*C* and *D*).

chromosomal translocations (Fig. 3*A*). Also note that there is a fully condensed chromosome 2 interchromosomal translocation in this cell, indicating that only a subset of the interchromosomal translocations induced by ionizing radiation display delay in mitotic chromosome condensation. A second metaphase spread with two chromosome 2 interchromosomal translocations that display delay in mitotic chromosome condensation is shown in Fig. 3*B*, and a spread with multiple chromosome 3 interchromosomal translocations that display delay in mitotic chromosome condensation is shown in Fig. 3, *C* and *D*. Note that some of the chromosome 3 interchromosomal translocations display delay in mitotic chromosome condensation, whereas others do not. Furthermore, an example of an extremely under-condensed chromosome that hybridized with the chromosome 2 whole chromosome paints is shown in Fig. 3, *E* and *F*. Note that this delay in mitotic chromosome condensation chromosome is so under-condensed that it cannot be scored for the presence or absence of translocations; yet, it is in a cell with two other chromosome 2 interchromosomal translocations. Taken together, these observations indicate that the chromosomes with the delay in mitotic chromosome condensation phenotype detected in primary blood lymphocytes 3 days after irradiation are predominantly interchromosomal translocations.

We detected 181 cells with interchromosomal translocations in a total of 1,178 metaphase spreads (Table 1). Given the known size of these chromosomes and the size of the human genome, we estimate that every cell in the irradiated population contained ~1 interchromosomal translocation (Table 1). The translocation frequency for genome equivalents was calculated using the method of Lucas *et al.* (11, 12). Therefore, in this study we found that exposing primary blood lymphocytes to 4 Gy of ionizing radiation produced chromosomes with delay in mitotic chromosome condensation in ~5% of the cells, and because delay in mitotic chromosome condensation occurred predominantly on interchromosomal translocations and every cell contained on average 1 interchromosomal translocation, our data suggest that ~5% of all interchromosomal translocations induced by ionizing radiation display the delay in replication timing/delay in mitotic chromosome condensation phenotype.

Ionizing Radiation Induces Chromosomes with Delay in Replication Timing/Delay in Mitotic Chromosome Condensation *In vivo*. To determine whether ionizing radiation can induce delay in mitotic chromosome condensation *in vivo* and to determine whether cells containing these chromosomes can persist for an extended period of time, we exposed mice to ionizing radiation and assayed mitotic spreads for chromosomes with delay in mitotic chromosome condensation. Mice, between 2 and 6 months of age, were exposed to 7.5 Gy of ionizing radiation on one-half of their body (6). Primary cultures of ear fibroblasts and kidney epithelial cells, from both irradiated and un-irradiated sides, were prepared at increasing times after ionizing radiation and analyzed for delay in mitotic chromosome condensation. No significant differences were observed between the kidney epithelial cells and the ear fibroblasts (data not shown), and, therefore, the

Table 1 *Number of cells with translocations after ionizing radiation*

Chromosome (WCP)	Size (bp × 10^8)	Fraction of genome	Total metaphases	Cells with translocations	Frequency of cells with translocations	Translocations per cell (estimated)
1	2.45	0.080	346	65	0.188	1.25
2	2.43	0.079	141	27	0.191	1.28
3	1.99	0.065	390	55	0.141	1.13
4	1.91	0.062	160	19	0.119	1.00
6	1.81	0.059	141	15	0.106	0.93
Total assayed	10.5	0.345	1178	181	NA	1.12

NOTE. Three days after exposure to 4 Gy of ionizing radiation, metaphase spreads from primary blood lymphocytes were hybridized with chromosomes 1 to 4 and 6 WCPs and scored for the presence of translocations. The translocation frequency for genome equivalents was calculated for each WCP using the method of Lucas et al. (11, 12). The fraction of the genome that each WCP covers is based on a haploid human genome size of 3.05×10^9 base pairs and the size of each chromosome (http://www.ensembl.org/Homo_sapiens). Taken together, there was an average of 1.12 translocations per whole genome equivalent.

Abbreviations: WCP, whole chromosome paint; NA, not applicable.

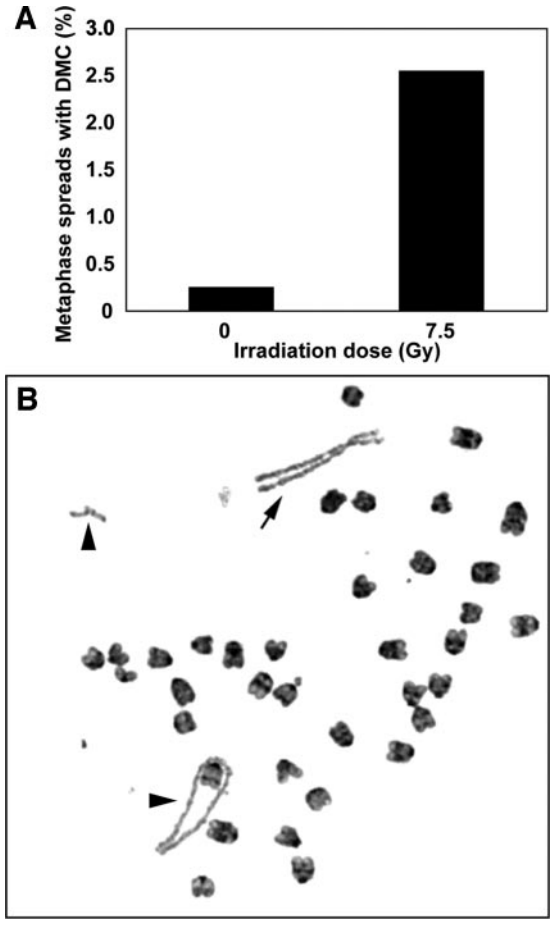

Fig. 4. Ionizing radiation causes DMC *in vivo* in mice. *A*, frequency of DMC in mouse primary cultures after ionizing radiation. DMC was detected in 2.5% (34 of 1,335) of metaphase spreads from irradiated tissues compared with only 0.25% (4 of 1,614) of metaphase spreads in unirradiated tissues. The number of spreads scored represents the sum of all metaphases harvested ranging from 3 days to 23 months after the mice were first exposed to ionizing radiation (see Materials and Methods). *B*, representative G-banded metaphase spread from a primary culture from an irradiated mouse ear. A translocated, metacentric chromosome with DMC (*arrow*) and two other DMC chromosomes (*arrowheads*) are shown. (DMC, delay in mitotic chromosome condensation)

data were combined. Delay in mitotic chromosome condensation was detected in 1.7% (8 of 474) of metaphase spreads prepared <1 month after exposure and in 2.6% (20 of 778) of metaphase spreads prepared between 12 and 24 months after exposure. Altogether, we detected delay in mitotic chromosome condensation in 2.2% of mitotic spreads from irradiated tissues and in 0.25% of mitotic spreads from unirradiated tissues (Fig. 4*A*). A representative metaphase spread from an irradiated culture is shown in Fig. 4*B*. Note that this spread has three different delay in mitotic chromosome condensation chromosomes, and one of them is a metacentric chromosome. Because all of the murine chromosomes are normally acrocentric, this metacentric chromosome must have experienced a structural rearrangement. These data indicate that exposing mouse cells to ionizing radiation *in vivo* induces chromosomes with delay in mitotic chromosome condensation and that cells containing these delayed chromosomes can persist for at least 2 years.

Chromosomes with Delay in Replication Timing/Delay in Mitotic Chromosome Condensation Are Associated with Hyperdiploid Karyotypes. During the analysis of the irradiated primary blood lymphocytes and the primary mouse cultures we noticed that the mitotic spreads containing chromosomes with delay in mitotic chromosome condensation were often hyperdiploid (ranging from tetraploid to >250 chromosomes; see Fig. 2*B* and Fig. 3, *C–F*). Analyzing the cells in the irradiated primary blood lymphocytes harvested only 3 days after irradiation indicated that ~33% of the metaphase spreads containing one or more delay in mitotic chromosome condensation chromosomes were hyperdiploid. In contrast, only ~3% of unirradiated metaphase spreads were hyperdiploid (Table 2). Similarly, analyzing the irradiated mouse cell cultures indicated that ~80% of irradiated spreads with one or more delay in mitotic chromosome condensation chromosomes and only ~16% of unirradiated spreads were hyperdiploid (Table 2). These observations indicate that the delay in replication timing/delay in mitotic chromosome condensation phenotype is associated with hyperdiploid karyotypes.

Engineering Chromosome Translocations with Delay in Replication Timing/Delay in Mitotic Chromosome Condensation. The results presented above suggest that ionizing radiation induces interchromosomal translocations with delay in replication timing/delay in mitotic chromosome condensation at a relatively high frequency. However, the exact nature of the chromosomal alterations responsible for the delay in replication timing/delay in mitotic chromosome condensation phenotype could not be determined. Therefore, we have developed a Cre/*lox*P chromosome engineering strategy that targets the genome in a random fashion, yet provides for the ability to track the chromosomes involved in the translocation events. A schematic representation of this strategy is shown in Supplemental Fig. 1. This strategy involves generating a collection of parental cell lines containing two, independently inserted, plasmid cassettes. One cassette contains the 5′ portion of the mouse *Aprt* gene linked to a Neomycin resistance gene (5′AP-Neo), and the other cassette contains the 3′ portion of the *Aprt* gene linked to a Hygromycin resistance gene (Hyg-3′RT). Each cassette contains *lox*P sites in the second intron of the *Aprt* gene. Thus, after Cre-mediated homologous recombination the *Aprt* gene is reconstituted at the *lox*P sites, and a reciprocal translocation is generated. This strategy uses a human cell line, HTD114, which is deficient for APRT (3). Note that HTD114 cells contain three pre-existing interchromosomal translocations (Supplemental Fig. 2) but display a relatively low frequency of spontaneous delay in replication timing/delay in mitotic chromosome condensation (<1%; see Fig. 1*B*).

For this analysis we isolated 54 independent parental clones containing random insertions of the two *lox*P cassettes. After transient transfection of each parental clone with a Cre expression plasmid and selection for *Aprt*-expressing cells, we recovered 10 independent recombinant cell lines. Because each colony within a parental clone is expected to generate the same translocation after any Cre-mediated event, we pooled the *Aprt*-expressing colonies from each parental clone to generate 10 recombinant pools. Each recombinant pool was analyzed for delay in mitotic chromosome condensation independently. This analysis identified a single recombinant pool (R27) with delay in mitotic chromosome condensation chromosomes.

To confirm that the delay in mitotic chromosome condensation was

Table 2 *DMC is associated with IR-induced hyperdiploid cells*

Cell type	Hyperdiploid	DMC	DMC and hyperdiploid
PBL			
No IR	2.8% (8/281)	0.14% (1/708)	0% (0/1)
IR	17.2% (274/1595)	5.5% (87/1595)	33.3% (29/87)
Mouse cells			
No IR	16.0% (258/1614)	0.24% (4/1614)	50% (2/4)
IR	20.8% (278/1335)	2.5% (34/1335)	79.4% (27/34)

NOTE. Metaphase spreads were scored as hyperdiploid if they contained tetraploid or greater numbers of chromosomes. The percentage of metaphase spreads with hyperdiploid karyotypes, DMC chromosomes, or hyperdiploid karyotypes with one or more DMC chromosome are shown. The actual numbers of metaphase spreads analyzed for each category is shown in parentheses.

Abbreviations: IR, ionizing radiation; DMC, delay in mitotic chromosome condensation.

occurring on the Cre-dependent translocation, we carried out a series of karyotypic studies on R27 and its respective parental clone (P27). In addition, we have carried out a similar analysis on a parental clone and its respective recombinant pool that did not generate chromosomes with delay in mitotic chromosome condensation. This analysis was carried out in the presence of Colcemid to facilitate the characterization of the chromosomes involved. Initially, we performed FISH analysis with the Aprt plasmid cassettes as probes to identify the sites of insertion and then used whole chromosome paints to confirm these insertion sites and to visualize the translocations generated. Finally, the translocations were verified using G-banding. P27 contains plasmid cassette insertions in chromosomes 3p13 and 16p13.3 (Fig. 5, *B–D*). R27 contains a new balanced translocation, t(3;16)(p13;p13.3), that was not present in P27 (Fig. 5, *B*, *E*, and *F*). A schematic representation of the balanced translocation generated in R27 is shown in Fig. 5*A*. To determine whether the t(3;16) present in R27

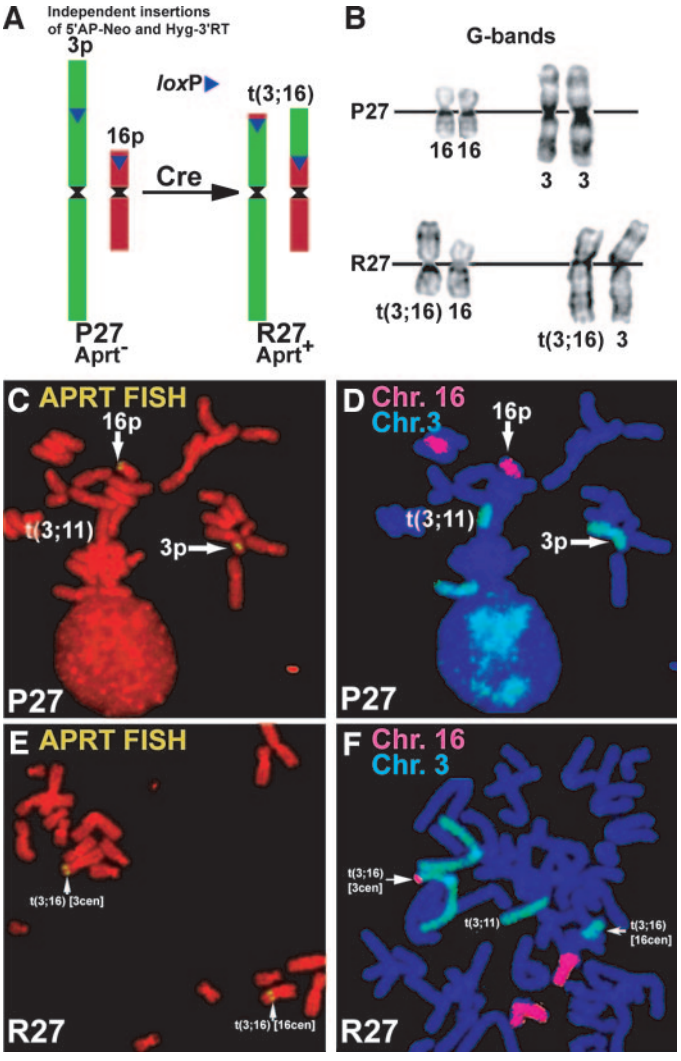

Fig. 5. Cytogenetic analysis of P27/R27. *A*, schematic diagram of the location of *lox*P insertion sites in P27. After Cre transfection, a reciprocal t(3;16) is generated. *B,* partial G-banded karyotypes identifying breakpoints from P27 and the t(3;16)(p13;p13.3) in R27. *C*, plasmid FISH, to detect the 5′AP-Neo and Hyg-3′RT cassettes, on a metaphase spread from P27. *D*, chromosome 3- and 16-specific whole chromosome paints confirm the location of the 5′AP-Neo and Hyg-3′RT plasmid inserts in P27. Note that there is a pre-existing t(3;11) in the HTD114 cell line (see Supplemental Data Fig. 2). *E*, plasmid FISH on a metaphase spread from R27 identifies a new t(3;16) reciprocal translocation. *F*, chromosome 3- and 16-specific whole chromosome paints confirm the presence of a t(3;16) translocation in R27. Note that the signal on the chromosome 16 derivative is interrupted by two overlying chromosomes. Chromosomes were counterstained with propidium iodide (*C* and *E*) or 4′, 6-diamidino-2-phenylindole (*D* and *F*).

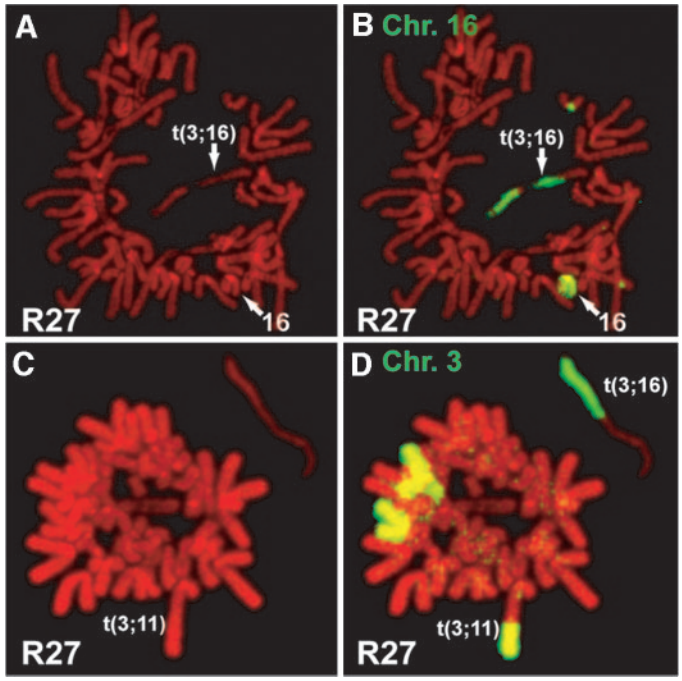

Fig. 6. Delay in mitotic chromosome condensation occurs on the engineered t(3;16) translocation. *A*, metaphase spread of R27 showing a delay in mitotic chromosome condensation chromosome. *B*, chromosome 16-specific whole chromosome paint confirms that this delay in mitotic chromosome condensation chromosome is a translocation. Note the normally condensed, nontranslocated chromosome 16. *C*, metaphase spread of R27 with a delay in mitotic chromosome condensation chromosome. *D*, chromosome 3-specific whole chromosome paint confirms that the delay in mitotic chromosome condensation chromosome in R27 is a translocation. The t(3;11) does not show delay in mitotic chromosome condensation and is also detected.

displays delay in mitotic chromosome condensation, we analyzed mitotic spreads, prepared in the absence of Colcemid, using FISH and chromosome 3 or 16 whole chromosome paints. Fig. 6, *A–D,* shows that these whole chromosome paints hybridized to delay in mitotic chromosome condensation chromosomes, indicating that the delay in mitotic chromosome condensation in R27 is occurring on the t(3;16). Finally, we have not observed delay in mitotic chromosome condensation on either chromosome 3 or chromosome 16 in P27, indicating that simple insertion of the plasmid cassettes at these particular locations is insufficient to cause delay in mitotic chromosome condensation (data not shown). A similar analysis of a parental clone and its recombinant pool that does not display delay in mitotic chromosome condensation is shown in the supplemental materials (Supplemental Fig. 3). These observations indicate that a subset of chromosome translocations generated by Cre/*lox*P-mediated homologous recombination display the delay in replication timing/delay in mitotic chromosome condensation phenotype.

DISCUSSION

One of the hallmarks of cancer is the presence of genetic instability. This instability can occur at distinct levels. In most cancers, the instability occurs at the chromosome level and is characterized by gains or losses of whole chromosomes (13). This chromosome instability is a dominant trait and is independent of p53 mutations (14). In addition, the frequent occurrence of chromosome translocations, marker chromosomes, and gene amplifications in many different types of tumors (1) suggests that a chromosomal instability also exists in cancer cells (13, 15). To distinguish between these two types of genomic instabilities, we refer to the process that results in gains or losses of intact whole chromosomes as chromosome instability and to

the process that results in the generation of frequent translocations and rearrangements as translocation instability.

Another well-documented example of genetic instability found in mammalian cells occurs at a delayed time after exposure to ionizing radiation (16, 17). This delayed or persistent chromosomal instability can occur *in vitro* or *in vivo* and is characterized by the appearance of new chromosome translocations and rearrangements for many generations after an initial exposure to ionizing radiation (18, 19). It is currently not known whether the translocation instability that is associated with cancer cells and the delayed chromosomal instability observed in irradiated cells are caused by similar or distinct mechanisms. However, we found previously that chromosomes with delay in replication timing/delay in mitotic chromosome condensation participate in frequent secondary translocations and rearrangements, suggesting that cells with delay in replication timing/delay in mitotic chromosome condensation display translocation instability (2). In this report, we show that chromosomes with delay in replication timing/delay in mitotic chromosome condensation are generated at a high frequency after exposure to ionizing radiation (>25% of surviving cells treated with 8 Gy) and that cells with delay in replication timing/delay in mitotic chromosome condensation chromosomes can persist for up to 24 days *in vitro* and up to 2 years *in vivo* after a single dose of ionizing radiation. Taken together, these observations indicate that the delay in replication timing/delay in mitotic chromosome condensation phenotype induced by ionizing radiation can persist for many generations and suggests that the translocation instability associated with cancer cells and the persistent chromosomal instability associated with ionizing radiation may be the consequence of the same process, namely delay in replication timing/delay in mitotic chromosome condensation.

One complicating factor in the studies described here is the previous observation that ionizing radiation induces the formation of micronuclei (20) and that these micronuclei contain late replicating DNA (21). Furthermore, these earlier studies also described late replicating, undercondensed chromosomes that were referred to as micronucleus-derived premature chromosome condensation. The micronucleus-derived premature chromosome condensation phenotype is distinct from the premature chromosome condensation that can be induced by the fusion of interphase and mitotic cells or by chemically induced premature mitosis (22, 23), because micronucleus-derived premature chromosome condensation affects only one or a few chromosomes instead of all of the chromosomes within a cell. The micronucleus-derived premature chromosome condensation was thought to be due to an interphase micronucleus undergoing premature chromosome condensation in response to the macronucleus entering mitosis (20, 21). However, because the micronucleus-derived premature chromosome condensation was observed in fixed metaphase spreads, it was not possible to determine whether or not these undercondensed chromosomes were in fact in micronuclei before mitosis. Regardless, this micronucleus-derived premature chromosome condensation appears to be very similar to the delay in replication timing/delay in mitotic chromosome condensation phenotype that we describe here and described previously for a subset of tumor-derived chromosome rearrangements (2). However, the tumor-derived chromosome rearrangements that display delay in replication timing/delay in mitotic chromosome condensation are not retained in micronuclei (2).[3] In addition, the t(3;16) with delay in mitotic chromosome condensation described in this study is also not retained in micronuclei (Supplemental Fig. 4). Therefore, late-replicating and undercondensed chromosomes can occur without being derived from micronuclei. In this report, we show that exposure of mammalian cells to ionizing radiation can induce a late-replicating and undercondensed chromosomal phenotype in as many as 25% of surviving cells and that this phenotype occurs predominantly on newly formed chromosome translocations. We believe that the ionizing radiation-induced micronucleus-derived premature chromosome condensation and the ionizing radiation-induced delay in replication timing/delay in mitotic chromosome condensation phenotypes represent the same or related phenomena. However, whether or not the ionizing radiation-induced late-replicating and undercondensed chromosomes were in micronuclei could not be determined from this or earlier studies. Thus, our Cre/*lox*P chromosome engineering data represent the first direct evidence that chromosome translocations are responsible for a late-replicating and undercondensed chromosomal phenotype and that these translocated chromosomes were not present within micronuclei. Therefore, our data support a model in which certain interchromosomal translocations are responsible for disrupting the normal replication timing of an entire chromosome and that this delayed replication results in delayed condensation during the subsequent mitosis, even when the chromosomes are retained within the same nucleus.

We found previously that introduction of a chromosome with the delay in replication timing/delay in mitotic chromosome condensation phenotype into karyotypically stable cells resulted in an aneuploid karyotype, *i.e.,* chromosome instability (24). In the present study, we found that cells containing chromosomes with delay in replication timing/delay in mitotic chromosome condensation induced by exposure to ionizing radiation often contained hyperdiploid karyotypes. Therefore, these observations indicate that chromosomes with delay in replication timing/delay in mitotic chromosome condensation are associated with two distinct types of genomic instability, translocation instability and chromosome instability. In addition, our results indicate that only a subset of chromosomal translocations induced by ionizing radiation, estimated to be ~5%, display the delay in replication timing/delay in mitotic chromosome condensation phenotype. Furthermore, ionizing radiation can induce delay in replication timing/delay in mitotic chromosome condensation in primary cells *in vitro* and *in vivo*, and the frequency at which ionizing radiation induces the delay in replication timing/delay in mitotic chromosome condensation phenotype is similar between immortalized cell lines and primary cells. For example, the frequency of delay in replication timing/delay in mitotic chromosome condensation in mitotic spreads harvested 3 days after 4 Gy of ionizing radiation was 5% in primary blood lymphocytes and 10% in GM639. Therefore, because the mutation frequency for single genes as well as the transformation frequency after exposure to this dose of ionizing radiation is expected to be much less than 5% (*i.e.,* $<1 \times 10^{-4}$; ref. 25), our data indicate that the delay in replication timing/delay in mitotic chromosome condensation phenotype can be induced in primary nontransformed cells. It should also be pointed out that smaller chromosomes are very difficult to score for delay in mitotic chromosome condensation. In addition, in cultures where 100% of the cells contained large chromosomes with delay in replication timing/delay in mitotic chromosome condensation, we could detect delay in mitotic chromosome condensation in <50% of the spreads (2). Therefore, we believe that our data represent an underestimate of the true frequency of the delay in replication timing/delay in mitotic chromosome condensation phenotype induced by ionizing radiation.

There are several models that can be formulated to explain the generation of chromosomes with the delay in replication timing/delay in mitotic chromosome condensation phenotype. In general, these models fall into two categories. First, it is possible that the delay in replication timing/delay in mitotic chromosome condensation phenotype is the result of a persistent epigenetic modification that occurs on

[3] L. Smith and M. Thayer, unpublished observations.

damaged chromosomes, perhaps as the result of a specific repair process. Second, it is possible that the delay in replication timing/delay in mitotic chromosome condensation phenotype is the result of a genetic alteration, perhaps the deletion of an element that promotes normal replication timing or the generation of an element that actively interferes with normal replication timing (2). Taken together, the data presented here indicate that this abnormal phenotype is regulated in *cis*-, and, therefore, may be under genetic control. Ionizing radiation is expected to generate between 20 and 40 DNA double-strand breaks per cell per Gy (25), whereas producing only 0.25 to 0.5 interchromosomal translocations per cell per Gy (9), indicating that the majority of double-strand breaks are repaired without the generation of interchromosomal translocations or the delay in replication timing/delay in mitotic chromosome condensation phenotype. Therefore, because we found that the majority of delay in mitotic chromosome condensation chromosomes induced by ionizing radiation were in fact interchromosomal translocations, but only 5% of interchromosomal translocations displayed delay in mitotic chromosome condensation, our data indicate that only a subset of the double-strand breaks that resolve into interchromosomal translocations produce the delay in replication timing/delay in mitotic chromosome condensation phenotype. Furthermore, we found that a subset of chromosome translocations (1 of 10) induced by Cre/*lox*P display this phenotype, whereas others do not. Therefore, our data indicate that the replication timing of an entire chromosome can be altered by a *cis*- mechanism. These data also suggest that the altered genetic material produced on certain chromosome translocations, rather than a simple epigenetic modification after DNA damage, is responsible for this abnormal chromosomal phenotype.

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