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Transmission of Genomic Instability from a Single Irradiated Human Chromosome to the Progeny of Unirradiated Cells

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Ionizing radiation can induce chromosome instability that is transmitted over many generations after irradiation in the progeny of surviving cells, but it remains unclear why this instability can be transmitted to the progeny. To acquire knowledge about the transmissible nature of genomic instability, we transferred an irradiated human chromosome into unirradiated mouse recipient cells by microcell fusion and examined the stability of the transferred human chromosome in the microcell hybrids. The transferred chromosome was stable in all six microcell hybrids in which an unirradiated human chromosome had been introduced. In contrast, the transferred chromosome was unstable in four out of five microcell hybrids in which an irradiated human chromosome had been introduced. The aberrations included changes in the irradiated chromosome itself and rearrangements with recipient mouse chromosomes. Thus the present study demonstrates that genomic instability can be transmitted to the progeny of unirradiated cells by a chromosome exposed to ionizing radiation, implying that the instability is caused by the irradiated chromosome itself and also that the instability is induced by the nontargeted effect of radiation.

INTRODUCTION

Ionizing radiation induces DNA double-strand breaks (DSBs), which play a crucial role in inducing biological effects including cell death, chromosome aberration, gene mutation and malignant transformation. However, it was recently found that biological effects emerge in the descendants of irradiated cells over many cell divisions, a finding that challenges the view that these effects are induced by the DSBs that are directly induced by radiation. Nontargeted radiation effects typically have been observed using delayed biological end points and are generally referred to as radiation-induced genomic instability (*1, 2*). Genomic instability contributes to an increased rate of genetic change and thus may be involved in multistep carcinogenesis (*3*). Therefore, gaining an understanding of the mechanism of genomic instability by radiation would provide an important clue to understanding the process of radiation carcinogenesis.

Chromosome instability is one of the most thoroughly studied delayed biological effect of ionizing radiation (*4–8*). It is well accepted that radiation-induced chromosome aberrations are formed by misrepair or incomplete repair of DSBs (*9*). Although the distinct molecular mechanism involved in the formation of chromosome aberrations is not yet clear, it is highly probable that abnormality is initiated by DSBs caused by the targeted effect of radiation and formed by the subsequent rejoining pathway governed mostly by non-homologous end joining. There is no evidence that delayed chromosomal instability is caused by a targeted effect of radiation. Rather, the most prominent feature of delayed chromosomal instability is that it is transmitted into the progeny of surviving cells, and thus direct exposure to radiation is not needed for the expression of the unstable phenotype. This implies that delayed chromosome instability might be induced by the nontargeted effect of radiation.

The possible mechanisms proposed for radiation-induced genomic instability include persistently elevated levels of reactive oxygen species (ROS) due to mitochondrial dysfunction (*10, 11*), altered chromatin organization due to large DNA deletions (*12*), and loss of function of telomeres (*13–15*). The fact that induced genomic instability can be transmitted through the progeny over many cell generations suggests the presence of a mechanism by which the initial DNA damage in the surviving cells is memorized. This transmitted memory causes delayed emergence of DSBs,

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which in turn activates the DNA damage checkpoint (*16*). However, there is no direct evidence that the unstable nature is conferred upon an irradiated chromosome by direct irradiation. If the unstable nature initiated by the targeted effect of radiation can be retained in the irradiated chromosome, then this would explain why the instability is transmissible to the progeny of surviving cells.

To gain knowledge about the transmissible nature of genomic instability, we transferred an irradiated human chromosome 11 into unirradiated mouse recipient cells by microcell-mediated chromosome transfer (*17*) and studied the stability of the introduced chromosome to find out whether the cause of instability resides in the irradiated chromosome itself. We discovered, and demonstrate here, that the irradiated chromosome is itself unstable and is capable of interacting with unirradiated chromosomes and rearranging over many cell generations.

MATERIALS AND METHODS

Cells and Cell Culture

Mouse A9 cells containing a single copy of human chromosome 11 were used as chromosome donors. Mouse m5S cells established from embryonic skin fibroblasts were used as recipients because they showed a stable near-diploid karyotype (*18, 19*). The m5S cells were cultured in modified α-minimum essential medium (α-MEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were maintained at 37°C in a humidified atmosphere of 95% air/5% CO_2.

Microcell Fusion

The donor cells were cultured in Dulbecco's modified Eagle's MEM (DMEM; Nissui Pharmaceutical Co., Tokyo, Japan) in 25-cm^2 flasks, and microcells were induced by treatment with 0.05 μg/ml Colcemid (Invitrogen) for 48 h in DMEM containing 20% FCS. The flasks were filled with serum-free medium containing 10 μg/ml cytochalasin B (Sigma-Aldrich, Co., St. Louis, MO), and then the microcells were isolated by centrifugation at 11,000 rpm for 30 min at 34°C. The crude microcells were purified by filtration through a series of polycarbonate filters with pore sizes of 8, 5 and 3 μm, resuspended in serum-free medium containing 100 μg/ml phytohemagglutinin (Sigma-Aldrich), and attached to the recipient m5S cells by incubation at 37°C for 15 min. The m5S cells were treated with 3 ml of polyethylene glycol (PEG) (Sigma-Aldrich) mixed with serum-free medium as 1:1.4 for 30 s, overlaid with 3 ml of a low-concentration PEG (PEG:serum-free medium, 1:3), and treated for another 40 s. After the m5S cells were washed three times with serum-free medium, they were fed with α-MEM containing 10% FCS. After incubation at 37°C for 24 h, the m5S cells were replated for selection in α-MEM containing 3 μg/ml blasticidin S (Funakoshi Co., Tokyo, Japan) for 2–3 weeks for colony formation. Blasticidin S-resistant microcell hybrids were isolated, grown for another 2 weeks in α-MEM supplemented with 3 μg/ml blasticidin S until almost reaching 10^6 cells, and then harvested for cytogenetic analysis. It was estimated that almost 20 cell divisions were occurred between colony isolation and cytogenetic analysis.

X Irradiation

Exponentially growing mouse A9 cells were irradiated with 4 or 6 Gy of X rays using a soft X-ray generator (Softex, Osaka, Japan) operating at 150 kVp and 5 mA with a 0.1-mm copper filter at a dose rate of 0.46 Gy/min. Immediately after irradiation, the cells were treated with 0.05 μg/ml colcemid for 48 h to induce microcells and were then subjected to microcell fusion as described above.

Chromosome Samples

Harvested metaphase cells were treated with hypotonic KCl (0.075 *M*) solution for 25 min at room temperature and fixed in fixative (methanol: acetic acid, 3:1). The mitotic cell suspensions were dropped onto a precleaned slide glass and dried for 24 h at room temperature.

Fluorescence In Situ Hybridization

The stability of the human chromosome 11 in the microcell hybrids was investigated by fluorescence *in situ* hybridization (FISH) using a fluorescent probe specific for a whole human chromosome 11 (whole chromosome painting-FISH; WCP-FISH). The chromosome slides were immersed in 2× SSC (0.3 *M* sodium chloride, 0.03 *M* sodium citrate, pH 7.0)/0.5% NP-40, incubated at 37°C for 30 min, and dehydrated by sequential rinse in 70%, 80% and 100% ethanol for 2 min each. Then they were immersed in 70% formamide/2× SSC for 4 min at 72°C to denature the chromosomes and dehydrated through a sequential rinse in the ethanol series described above. An aliquot (6 μl) of a fluorescent probe (Q-Biogene, Montreal, Canada) was applied on the slides, and the slides were then covered with glass cover slips, sealed with rubber cement, and incubated for 12–16 h in a humidified chamber at 37°C. After hybridization, the slides were rinsed in 50% formamide/2× SSC for 15 min at 43°C, washed in 0.1× SSC for 15 min at 60°C, rinsed in 1× PBD buffer (Q-Biogene) for 10 min at room temperature, and stained with 10 μl of DAPI (Vysis Inc., Downers Grove, IL) in antifade. The metaphase chromosomes were visualized under a fluorescence microscope (Olympus Co., Tokyo, Japan), and digital images were recorded using a CCD camera (Photometrics).

Detection of γ-H2AX Foci

Foci of phosphorylated histone H2AX (γ-H2AX) in microcell hybrids were visualized by an immunofluorescence method described previously (*20*). Briefly, cells grown on cover slips were washed once with cold Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline [PBS(−)], fixed with 4% formalin/PBS(−) solution for 10 min at room temperature, and permeabilized with 0.5% Triton X-100/PBS(−) solution for 5 min on ice. Cells were incubated for 2 h at 37°C with mouse monoclonal anti-phosphorylated H2AX at Ser139 antibody (Upstate Biotechnology, Inc., Stony Brook, NY). After the cells were washed three times with PBS(−), they were incubated for 1 h at 37°C with Alexa Fluor 488-conjugated goat anti-mouse antibody (Molecular Probes, Inc., Eugene, OR). Then the nuclei were counterstained with 10 ng/ml DAPI for 30 min, and cover slips were mounted on slide glasses with PBS(−) containing 10% glycerol. Images were acquired with a fluorescence microscope (Olympus).

RESULTS

Stability of an Unirradiated Human Chromosome 11 in the Microcell Hybrids

We transferred an unirradiated human chromosome 11 into the unirradiated mouse m5S cells by microcell fusion, isolated the microcell hybrids, and then examined the stability of the transferred human chromosome in six microcell hybrids, 2011-4, 2011-13, 2011-14, 5011-14, 5011-15 and 5011-18, using WCP-FISH. More than 92% of the microcell hybrids examined remained near-diploid cells, with one copy of human chromosome 11 per near-diploid cell (Table 1). Less than 4% of the cells of three microcell hybrids (2011-4, 13 and 14) had a structural abnormality of

TABLE 1
Numerical Chromosome Changes in Microcell Hybrids

Cells	Dose (Gy)	No. of cells scored	Percentage of cells		Copy numbers of chromosome 11 per cell	
			Near-diploid	Tetraploid	Near-diploid	Tetraploid
2011-4	0	126	100	0	1.00 ± 0.01	
2011-13	0	200	100	0	1.01 ± 0.01	
2011-14	0	128	100	0	0.99 ± 0.01	
5011-14	0	100	92	8	1.01 ± 0.10	2.00 ± 0.00
5011-15	0	100	94	6	1.00 ± 0.00	2.00 ± 0.00
5011-18	0	100	95	5	1.00 ± 0.00	2.00 ± 0.00
5X11-1	4	100	98	2	1.02 ± 0.14	2.00 ± 0.00
5X11-9	4	100	99	1	1.04 ± 0.20	3.00
5X11-13	4	100	96	4	1.00 ± 0.00	2.00 ± 0.00
5X11-50	4	100	89	11	1.00 ± 0.00	2.00 ± 0.00
6X11-11	6	196	97.4	2.6	1.02 ± 0.18	1.80 ± 0.44

the human chromosome 11 before and after chromosome transfer (Table 2). No abnormities were detected in the remaining three microcell hybrids (5011-14, 15, and 18) (Table 2). The fact that the transferred human chromosome remained stable in the microcell hybrids indicates that this experimental system using chromosome transfer is suitable for evaluating the nontargeted radiation effect on chromosome stability.

Stability of an Irradiated Human Chromosome 11 in the Microcell Hybrids

We examined the stability of X-irradiated human chromosome 11 in five microcell hybrids, 5X11-1, 5X11-9, 5X11-13, 5X11-50 and 6X11-11. The five microcell hybrids retained near-diploid karyotypes in more than 89% of cells examined, as shown in Table 1. The copy number of chromosome 11 was almost one per near-diploid cell, which was similar to that observed in the microcell hybrids into which an unirradiated chromosome 11 had been introduced (Table 1). In contrast to the unirradiated chromosome, the irradiated human chromosome 11 was unstable in four out of five microcell hybrids (Table 2). For example, as shown in Fig. 1, the 5X11-9 cells exhibited eight different types of aberrations, including deletion of a short arm (panel b, 29%), isochromosome formation (panel c, 24%), translocation with a recipient mouse chromosome (panel d, 11%), Robertsonian-type translocation with a mouse chromosome (panel e, 4%), ring (panel f, 2%), fragment (panel g, 2%), and two types of complicated intra-rearrangement (panel h, 9%; panel i, 8%). The rearranged chromosomes in the 5X11-1 cells and the 5X11-50 cells (panel j, 38%) were mostly fragments (62%, data not shown), as shown in Fig. 1. The 6X11-11 cells showed five types of aberration, including translocations (two types) with a recipient mouse chromosome (45.4%), rings (25.5%), and deletions (two types, 1%). These results indicate that the irradiated chromosome 11 is unstable and further suggest that radiation-induced lesions that trigger subsequent rearrangements involving the irradiated chromosome remain at long times after exposure.

Phosphorylated Histone H2AX Foci in Microcell Hybrids

To examine the possibility that residual radiation lesions remain in irradiated human chromosome 11, we investigated the number of foci of phosphorylated histone H2AX (γ-H2AX) in interphases of microcell hybrids containing an irradiated human chromosome 11. In the 2011-4 and

TABLE 2
Stability of Human Chromosome 11 in Microcell Hybrid Cells

Cells	Dose (Gy)	No. of cells scored	Transferred chromosome 11	No. of aberration types observed	No. of cells with rearranged chromosome 11 after chromosome transfer (%)	No. of cells with altered copy no. of chromosome 11 after chromosome transfer (%)
2011-4	0	126	intact	1	5 (4)	2 (1.6)
2011-13	0	200	intact	2	2 (1)	2 (1)
2011-14	0	128	intact	1	1 (0.8)	1 (0.8)
5011-14	0	100	intact	0	0 (0)	1 (1)
5011-15	0	100	intact	0	0 (0)	0 (0)
5011-18	0	100	intact	0	0 (0)	0 (0)
5X11-1	4	100	intact	2	62 (62)	2 (2)
5X11-9	4	100	intact	8	85 (85)	7 (7)
5X11-13	4	100	intact	0	0 (0)	0 (0)
5X11-50	4	100	intact	1	38 (38)	2 (2)
6X11-11	6	196	intact	5	141 (72)	2 (1)

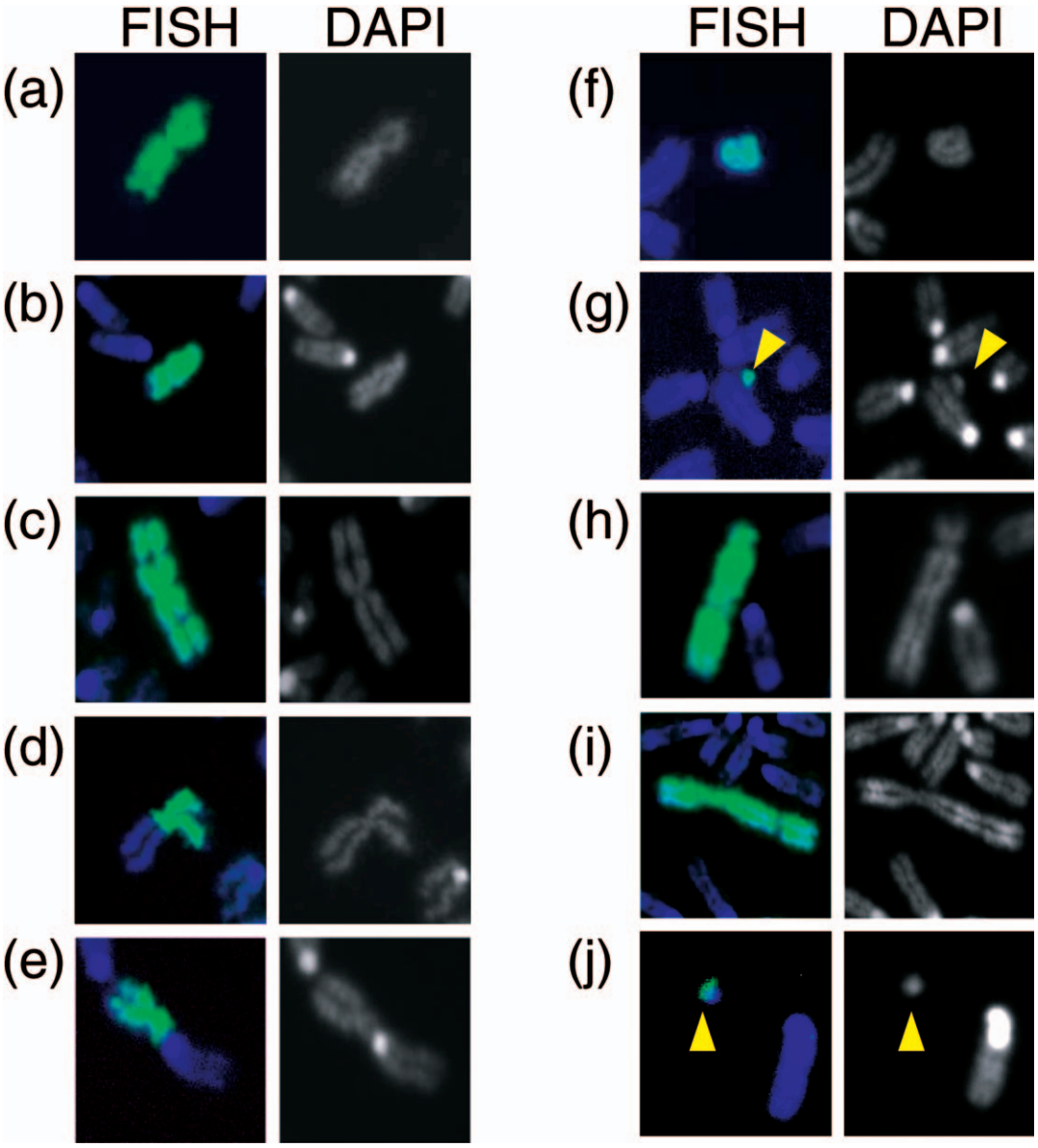

FIG. 1. Instability of a human chromosome 11 in microcell hybrids 5X11-9 (panels a–i) and 5X11-50 (panel j). Intact and rearranged human chromosomes 11 were visualized by FISH and all chromosomes were counterstained with DAPI. Panel a: Intact human chromosome 11, 15%; panel b: deletion of a short arm of chromosome 11, 29%; panel c: isochromosome 11, 24%; panel d: translocation with a recipient mouse chromosome, 11%; panel e: Robertsonian-type translocation with a mouse chromosome, 4%; panel f: ring chromosome 11, 2%; panel g: fragment of chromosome 11, 2%; panels h and i: complicated intra-rearrangement of chromosome 11, 9% and 8%, respectively; panel j: fragment of chromosome 11, 38%. Arrowheads indicate the fragment of chromosome 11.

2011-13 cells, both of which contained an unirradiated chromosome 11, the average number of foci per cell was 2.0. In the 6X11-11 cells, in which chromosome 11 had been exposed to 6 Gy of X rays and was highly unstable (72% of the cells showed evidence of instability), the number of foci per cell was 1.9. Thus we failed to find a difference in the number of foci of γ-H2AX between microcell hybrids with an unirradiated chromosome and those with an irradiated chromosome. In other words, we could not detect any lesions produced by the direct effects of radiation.

Stability of Human Chromosome 11 in the Isolated Secondary Microcell Hybrids

To find out whether the instability of the irradiated chromosome is transmitted to the progeny of microcell hybrid cells, we isolated secondary colonies from 5X11-9 cells and analyzed the stability of the human chromosome 11 in six clones derived from these secondary clones. It was estimated that an additional 20 cell divisions occurred between secondary colony isolation and cytogenetic analysis.

As shown in Table 3, all cell clones retained their original ploidy (near-diploid) and copy number of human chro-

TABLE 3
Numerical Chromosome Changes in Secondary Colonies Derived from 5X11 Cells

Cells	Dose (Gy)	No. of cells scored	Percentage of cells		Copy numbers of chromosome 11 per cell	
			Near-diploid	Tetraploid	Near-diploid	Tetraploid
5X11-9A	4	100	86	14	1.06 ± 0.24	2.07 ± 0.73
5X11-9B	4	50	92	8	1.02 ± 0.15	2.50 ± 0.58
5X11-9C	4	50	95	5	1.33 ± 0.60	2.00 ± 0.00
5X11-9D	4	50	100	0	1.06 ± 0.24	
5X11-9E	4	50	100	0	1.06 ± 0.24	
5X11-9F	4	50	95	5	1.00 ± 0.00	2.60 ± 0.89

mosome 11 (one per diploid). Four of six cell clones retaining an isochromosome 11 showed no further rearrangement after recloning (Table 4). Clone 5X11-9C, which contained a complicated aberrant chromosome 11 (deletions of both arms of chromosome 11), showed a low frequency (4%) of rearrangement after recloning (Table 4). In contrast, clone 5X11-9A was unstable. In that clone, human chromosome 11 showed six different types of aberrations at high frequency (99%), as shown in Table 4 and Fig. 2. This indicates that the unstable nature of an irradiated chromosome can be transmitted in the progeny of unirradiated cells over many cell divisions.

DISCUSSION

In the present study, we examined the stability of single irradiated human chromosomes transferred into unirradiated mouse recipient cells. We found that instability can be transmitted via the directly exposed, transferred chromosome to the clonal progeny of unirradiated cells, clearly demonstrating that the induced instability arises from the irradiated chromosome. In addition to intrachromosomal aberrations, interchromosomal aberrations between the irradiated human chromosomes and unirradiated mouse chromosomes were also evident. These results suggest that the irradiated chromosome itself enhances the potential for genomic rearrangement to occur, promoting interactions with unirradiated chromosomes.

Radiation-induced genomic instability has been demonstrated in studies on mutation and chromosome aberrations that emerge in cells surviving radiation exposure (*1, 2*). However, the mechanism underlying these phenomena is not well understood. In terms of mutagenesis, the molecular structural spectrum of the mutants that emerge in cells more than 30 generations postirradiation (nontargeted mutation) is similar to those of spontaneous mutants that are predominantly point mutations (*21, 22*). This suggests that these nontargeted mutants are induced by a mechanism different from that inducing targeted mutants that include predominantly deletions. When chromosome aberrations occur, dicentric chromosomes formed by the targeted effect of radiation are accompanied primarily by fragments. In contrast, most dicentrics that arise in the cells more than 30 generations postirradiation (nontargeted aberrations) are not accompanied by fragments (*23*). We previously showed that these types of dicentrics without fragments are formed by telomeric end-to-end fusions induced by the nontargeted effect of radiation (*13, 15*). This again suggests the possibility that the pathway leading to the formation of nontargeted aberrations differs from that for targeted aberrations.

These findings imply that DSBs, which are produced by the targeted effect of radiation, are not themselves the main

TABLE 4
Stability of Human Chromosome 11 in Secondary Colonies Derived from 5X11 Cells

Cells	Dose (Gy)	No. of cells scored	Transferred chromosome 11	No. of aberration types observed	No. of cells with rearranged chromosome 11 after chromosome transfer (%)	No. of cells with altered copy no. of chromosome 11 after chromosome transfer (%)
5X11-9A	4	100	intact	6	99[a] (99)	11 (11)
5X11-9B	4	50	i(11)[b]	1	0 (0)	3 (6)
5X11-9C	4	50	Compli[c]	2	2 (4)	12 (24)
5X11-9D	4	50	i(11)[b]	1	0 (0)	3 (6)
5X11-9E	4	50	i(11)[b]	1	0 (0)	3 (6)
5X11-9F	4	50	i(11)[b]	1	0 (0)	0 (0)

[a] One metaphase included both an intact chromosome 11 and a dicentric chromosome 11, resulting in 99% of the cells containing rearranged chromosome 11 despite the fact that 2% of the cells contained an intact chromosome 11, as shown in Fig. 2.

[b] Isochromosome 11.

[c] Complicated intra-rearrangement.

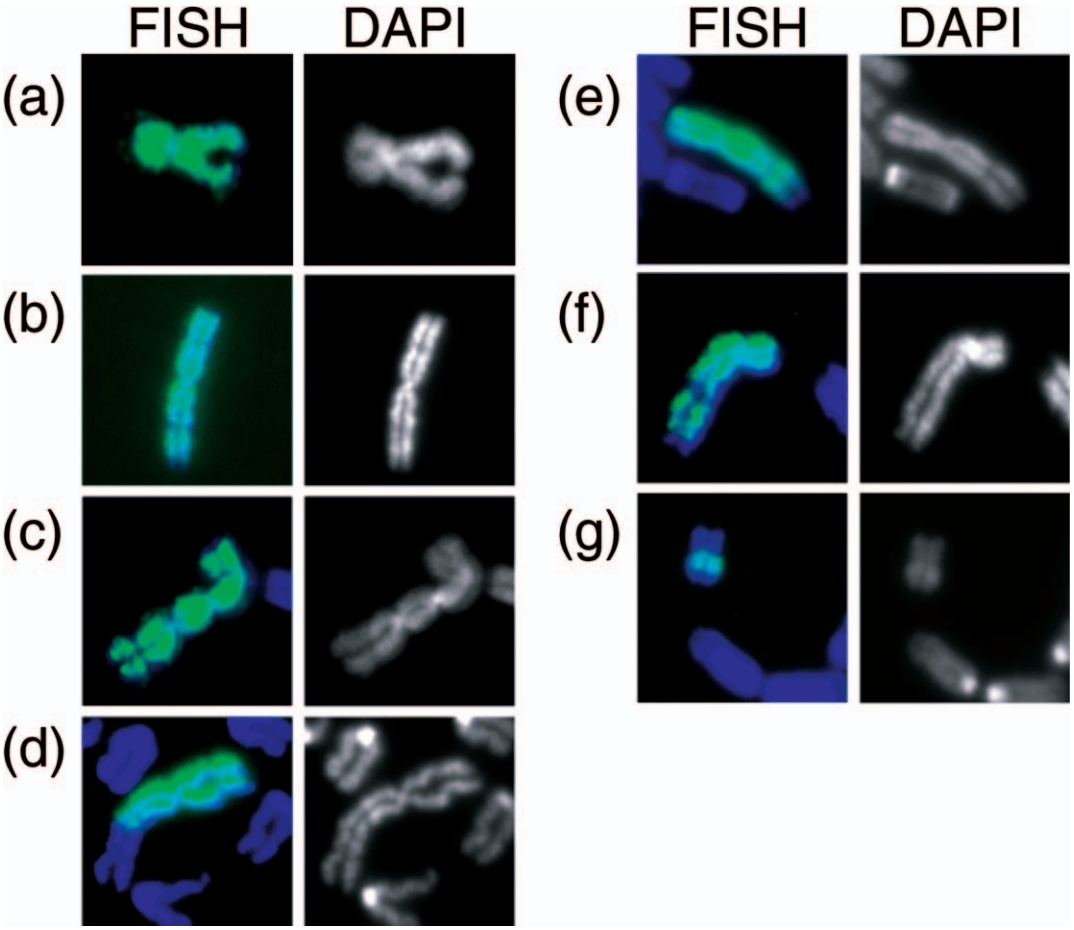

FIG. 2. Instability of a human chromosome 11 in a secondary microcell hybrid 5X11-9A. Intact and rearranged human chromosomes 11 were visualized by FISH and all chromosomes were counterstained with DAPI. Panel a: Intact human chromosome 11, 2%; panel b: isochromosome 11, 91%; panel c: dicentric chromosome 11, 3%; panel d: translocation with a recipient mouse chromosome, 2%; panels e and f: complicated intra-rearrangement of chromosome 11, 2% and 1%, respectively; panel g; fragment of chromosome 11, 1%.

cause of genomic instability. Rather, we speculate that some of the unstable lesions that remain in a chromosome after DSB repair is completed may be responsible for the induction of genomic instability. To examine whether the presence of these residual lesions could enhance instability in a chromosome exposed to radiation, we introduced an irradiated human chromosome into unirradiated mouse cells. In our protocol for chromosome transfer, the irradiated chromosome-donor cells were incubated for 48 h to allow the formation of microcells, and thus a majority of DSBs should have been rejoined before chromosome transfer. Therefore, unrejoined DSBs are not the main cause of the instability of the irradiated chromosomes in recipient cells.

Evidence has been accumulating that formation of γ-H2AX foci is a biological marker of DSBs, because it responds to radiation in a sensitive and dose-dependent manner (*24*). We failed to show a difference in the number of foci of γ-H2AX between microcell hybrids with an unirradiated chromosome and those with an irradiated chromosome. This indicates that there are no long-lived foci at long times after exposure to radiation, presumably due to repair of such lesions before these late observations.

The fact that an irradiated chromosome is less stable than an unirradiated chromosome indicates that an irradiated chromosome retains potentially active sites that enhance the possibility of genomic rearrangement. However, we have no information to indicate that the active site is located at the same site where a primary break arises. In addition, the fact that not all microcell hybrids with the irradiated chromosome exhibited instability and that only one of the secondary clones was unstable supports the idea that there is individual variability in the development of instability.

In the exchange theory proposed by Revell (*25*) for the origin of chromosome aberrations, the primary event of damage is not a break induced directly by radiation but an unstable lesion that decays to a stable, undetectable state if the secondary event, an exchange process, does not follow. Our concept of ''the residual unstable lesion'' and that of Revell's ''primary event of damage'' are not identical, but they do share the basic idea that hot spots emerge that promote genomic rearrangement in an irradiated chromosome. Our results demonstrating elevated chromosomal instability in a secondary colony derived from an unstable microcell hybrid suggest that at least in some instances, the unstable

lesion persists over many cell divisions, in contrast to the Revell hypothesis, where the primary damage event tends to return toward a stable state.

The intriguing question becomes how the experience of exposure to radiation is remembered by cells. We previously demonstrated that radiation destabilized telomere function, which promoted persistent chromosome instability in immortalized mouse (*13, 14*) and human cells (*15*), suggesting that telomeres may be susceptible to the induction of genomic instability. Therefore, we speculate that telomere destabilization is a possible mechanism by which the memory of irradiation is retained. Evidence that the loss of a single telomere can result in the instability of multiple chromosomes has been well documented in studies using human tumor cells (*26–28*). Telomere destabilization does not induce immediate cell death, but it increases the probability of evoking chromosome instability over many cell divisions. Thus the hypothesis that radiation induces telomere dysfunction may well explain the unstable nature of the irradiated chromosomes in the chromosome transfer experiments in the present study.

In summary, we have demonstrated that an irradiated chromosome can transmit its acquired instability to the progeny of unirradiated cells, implying that the memory of the radiation event is retained in the exposed chromosome, and thus the effect of radiation in inducing delayed chromosome instability is nontargeted.

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