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Authors: Nagasawa, H., Peng, Y., Wilson, P. F., Lio, Y-C., Chen, D. J., et al.

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Role of Homologous Recombination in the Alpha-Particle-Induced Bystander Effect for Sister Chromatid Exchanges and Chromosomal Aberrations

H. Nagasawa,[a] Y. Peng,[a] P. F. Wilson,[a] Y-C. Lio,[b] D. J. Chen,[b] J. S. Bedford[a] and J. B. Little[c,1]

[a] *Department of Environmental and Radiological Health Sciences, Colorado State University, Fort Collins, Colorado 80523;* [b] *Department of Radiation Oncology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75390; and* [c] *Center for Radiation Sciences and Environmental Health, Harvard School of Public Health, Boston, Massachusetts 02115*

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The bystander effect for sister chromatid exchanges (SCEs) and chromosomal aberrations was examined in hamster cell lines deficient in either DNA-PKcs (V3 cells, deficient in nonhomologous end joining, NHEJ) or RAD51C (*irs3* cells, deficient in homologous recombination, HR). Cells synchronized in G_0/G_1 phase were irradiated with very low fluences of α particles such that <1% of the nuclei were traversed by an α particle. Wild-type cells showed a prominent bystander response for SCE induction; an even greater effect was observed in V3 cells. On the other hand, no significant induction of SCE was observed in the *irs3* RAD51C-deficient bystander cells irradiated at various stages in the cell cycle. Whereas a marked bystander effect for chromosomal aberrations occurred in V3 cells, the induction of chromosomal aberrations in *irs3* bystander cells was minimal and similar to that of wild-type cells. Based on these findings, we hypothesize that HR is essential for the induction of SCE in bystander cells; however, HR is unable to repair the DNA damage induced in NHEJ-deficient bystander cells that leads to either SCE or chromosomal aberrations.

INTRODUCTION

The important cellular effects of exposure to ionizing radiation arise primarily from the induction of DNA double-strand breaks (DSBs) as a consequence of the nature of the energy deposition in DNA by ionizations that produce local clustered damage (*1*). Such DSBs can be repaired in mammalian cells by two recombinatorial pathways, nonhomologous end joining (NHEJ) and homologous recombination (HR) (*2, 3*). NHEJ is active throughout the cell cycle (predominating in G_0/G_1-phase cells) and is an error-prone process that does not require extensive regions of homology at the site of repair. Repair of DSBs by homologous recombination, on the other hand, is an error-free mechanism that occurs primarily in late S and G_2 phases of the cell cycle; HR uses homologous chromosome regions (the sister chromatid, in this case) as a template for repair. While the importance of homologous recombinational processes is clear and, in fact, essential (full knockout of many genes involved with HR is embryonic lethal), the relative contribution of HR to the processing and repair of DNA damage induced directly by ionizing radiation is still not clear (*4*).

In earlier studies, we reported a prominent bystander effect for the induction of sister chromatid exchanges (SCEs) after α-particle irradiation. Approximately 30% of the cells in confluent cultures showed increased levels of SCEs when 1% or fewer were traversed by an α particle (*5*). The bystander effect for the induction of *HPRT* mutations or chromosomal aberrations in wild-type cells was much smaller (*6, 7*). Interestingly, the mutations induced in bystander cells were almost entirely point mutations, in contrast to directly irradiated cells, in which total and partial gene deletions predominated (*8*). NHEJ-deficient cells, however, showed a very marked bystander effect for both chromosomal aberrations and *HPRT* mutations; mutations in these cells consisted primarily of deletions (*7, 9*). We hypothesize that the mutations induced in wild-type bystander cells are largely the result of oxidative base damage (*10*). While very few DSBs are produced by various reactive oxygen species (ROS), generated either directly by radiation or indirectly through a signal transduction process (*10*), they can arise from clustered lesions and may be increased transiently in irradiated normal cells at replication forks (*11*). Normally these DSBs are effectively repaired, but in NHEJ-deficient cells they are not, rendering bystander cells in these cultures more sensitive to the induction of large-scale mutations and chromosomal aberrations.

Since HR is known to play an essential role in the maintenance of genomic integrity, the present study was de-

[1] Address for correspondence: Building 1, Room 505, 665 Huntington Avenue, Boston, MA 02115; e-mail: jlittle@hsph.harvard.edu.

TABLE 1
Cell Lines and Radiosensitivity

	Cell line			
	CHO	V3/vec	V-79	IRS-3
Radiosensitivity				
D_0 (Gy)	1.96	0.4	1.75	1.90
D_{10} (Gy)	5.41	1.05	5.50	4.20
DNA-PKcs	Wild-type	Mutant	Wild-type	Wild-type
RAD51C	Wild-type	Wild-type	Wild-type	Mutant

signed to examine the potential role of HR in the bystander effect. We examined the induction of chromosomal aberrations and specifically SCEs, since SCEs are thought to be a consequence of HR processes in which one possible outcome can be a crossover at the damage site resulting in the exchange of sister chromatids (*12–14*). We employed a hamster cell line deficient in *RAD51C*, a *RAD51* paralog known to be involved in HR and Holliday junction processing in human cells (*15*).

MATERIALS AND METHODS

Cells and Cell Culture

The *irs3* cell line is derived from the wild-type V79 (Chinese hamster lung) fibroblast cell line and is characterized by a mutation in the *RAD51L2* gene and undetectable levels of RAD51L2 protein (RAD51C) (*16*). V3 cells are an extremely radiosensitive cell line derived from CHO (Chinese hamster ovary) cells (*17*). Although the precise nature of the mutation in V3 cells is unknown, they have no measurable level of DNA-PKcs protein (*18*), and wild-type radiosensitivity is completely restored by transfection of *PRKDC* [the gene encoding DNA-PKcs (*19*)]. V3/vec cells are V3 cells transfected with an empty vector rather than those transfected with the *PRKDC*-containing vector (*19*). Cell lines were cultured at 37°C in a humidified 95% air/5% CO_2 atmosphere with Eagle's minimal essential medium (MEM, Gibco, Grand Island, NY) supplemented with 10% heat-inactivated (56°C for 30 min) fetal bovine serum (FBS, Hyclone, Logan, UT), 50 U/ml penicillin, and 50 μg/ml streptomycin (Sigma, St. Louis, MO). The mean doubling times of all the cell lines examined were approximately 12 ± 1 h. To achieve cell synchrony, cells were seeded in growth medium at a density of 10^5 cells on specially designed stainless steel dishes with a 1.5-μm-thick Mylar base (3.8 cm in diameter, 11.3 cm^2 surface area) coated with fibronectin to facilitate cell attachment. After overnight culture, the culture medium was replaced with isoleucine-deficient MEM containing 5% 3× dialyzed FBS and antibiotics to synchronize cells in G_0/G_1 phase. Isoleucine-deficient medium was changed twice at 24-h intervals. Irradiation experiments were initiated 30 h after the second medium change when the cultures reached ~30–50% confluence. Radiosensitivity, as measured by clonogenic survival after exposure to graded doses of ^{137}Cs γ radiation, was measured by standard techniques as described previously (*20*).

Immunoblots

Whole cell extracts were prepared in cell lysis and extraction buffer as described previously (*21*). Protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IL) was added to this buffer. The protein concentration in these extracts was determined by the BCA protein assay kit (Pierce, Rockford, IL); 50 μg of total protein was resolved by 4–12% SDS-PAGE (Invitrogen, Carlsbad, CA) and transferred onto nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). The membrane was then blocked with 7% dry milk in phosphate-buffered saline (PBS). The DNA-PKcs protein was detected by a mouse antibody (Ab4, Neomarkers, Fremont, CA) and the RAD51C protein was detected with a rabbit antibody (clone 2H11, Chemicon, Temecula, CA). β-Actin served as the total protein loading control and was detected using a mouse anti-β-actin monoclonal antibody (NB 600-501, Novus, Littleton, CO). Secondary detection was achieved with horseradish peroxidase-labeled goat anti-mouse or goat anti-rabbit IgG (Sigma). Antibody-labeled proteins were visualized by ECL reagent (Amersham Biosciences) and exposure to X-ray film (Kodak, Rochester, NY).

Alpha-Particle Irradiation

For α-particle irradiations, the Mylar-based culture dishes were placed in the exposure well of a benchtop α-particle irradiation system described previously (*22*). This system consists of approximately 275 MBq of $^{238}PuO_2$ electroplated on a 10-cm-diameter stainless steel disk enclosed in a plexiglass box filled with helium at ambient air pressure. The cells were irradiated from below by α particles traversing a rotating collimator and the sealed 1.5-μm-thick Mylar window of the exposure well. The dose delivered to cells is controlled by a timer and a photographic shutter system, allowing precise irradiation times to achieve doses as low as 0.01 cGy with high accuracy. Revised dosimetric measurements of this system yield a calculated dose rate of 8.3 cGy/min and an α-particle fluence of 0.47 track/nucleus min^{-1} (average LET = 113 keV/μm) for CHO-sized cells (*23*). Alpha-particle spectroscopy and CR-39 track-etch measurements provide values of 3.86 MeV for the average α-particle energy at the cell surface and a fluence of 0.0045 particle/μm^2 min^{-1}. The dose rate and LET were obtained by Monte Carlo transport code and energy transport calculations (manuscript in preparation).

Chromosome Analysis

To measure chromosomal aberrations and SCEs, the culture medium was replaced with MEM/10% FBS medium containing 10^{-5} *M* 5′-bromodeoxyuridine (BrdU, Sigma) immediately after α-particle irradiation. The Mylar-based culture dishes were returned to the incubator to allow the synchronized cells to progress into the first and second postirradiation mitoses. To measure chromosomal aberrations in the first postirradiation mitosis and SCE in the second postirradiation mitosis, 0.2 μg/ml Colcemid (GIBCO, Grand Island, NY) was added to each dish for three successive 6-h intervals beginning 10 h after irradiation. Metaphase chromosome spreads were collected and prepared by the air-dry method, and differential staining of the chromosomes was obtained by the fluorescence plus Giemsa technique (*24*). Both chromosome and chromatid-type aberrations in the first postirradiation mitosis were scored. SCEs in the second postirradiation mitosis were scored as described previously (*5*).

RESULTS

Table 1 summarizes the radiosensitivities as measured by clonogenic survival for the four cell lines examined in this

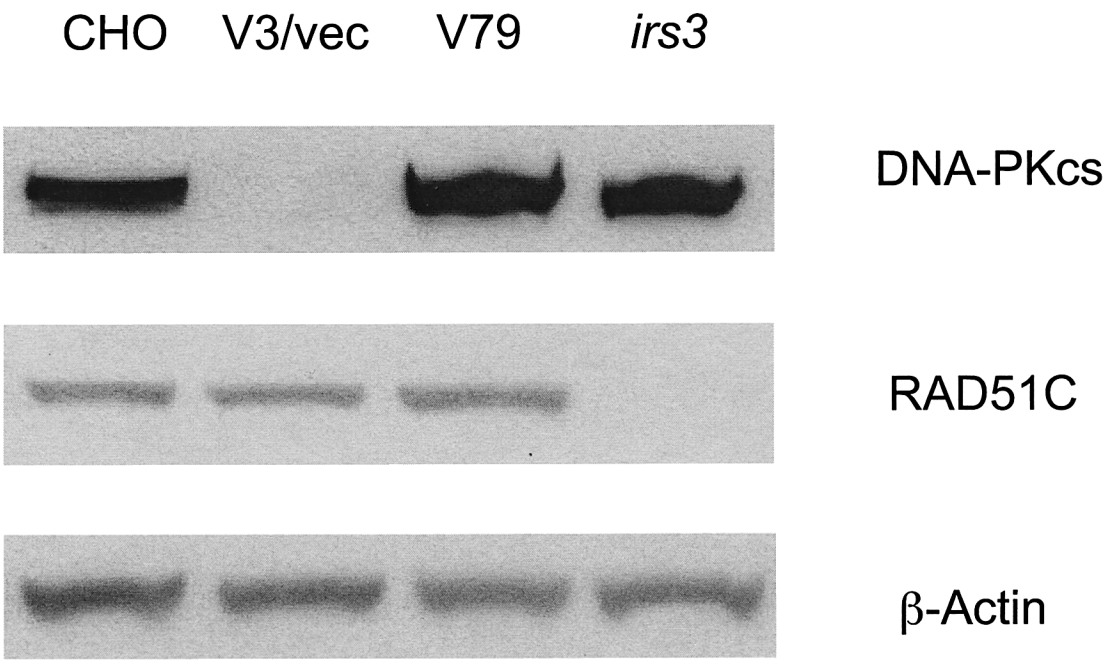

FIG. 1. Western immunoblot analysis of DNA-PKcs and RAD51C protein expression in wild-type CHO and V79 cells and V3/vec and *irs3* cells.

study, along with their corresponding *PRKDC* (DNA-PKcs) and *RAD51C* genotypes. The V3/vec line is a clonal derivative of the V3 cell line, an NHEJ-deficient line originally derived from CHO cells (*19*). As confirmed by the immunoblot analysis shown in Fig. 1, V3/vec cells are deficient in DNA-PKcs. The HR-deficient line *irs3* was derived from wild-type V79 cells (*16*), and the RAD51C deficiency is also confirmed by the immunoblot analysis shown in Fig. 1. As is characteristic for other cell lines deficient in NHEJ, the V3/vec cells are highly radiosensitive with respect to cell killing (Table 1) and are also hypersensitive for induction of chromosomal aberrations (see Fig. 4). On the other hand, the *irs3* cells defective in HR showed no increased sensitivity for either end point of radiation damage.

Figure 2A and B shows the results of experiments measuring the induction of SCE in CHO and NHEJ-defective V3/vec cells (Fig. 2A) and V79 and HR-defective *irs3* cells (Fig. 2B) by very low fluences of α particles. The mean

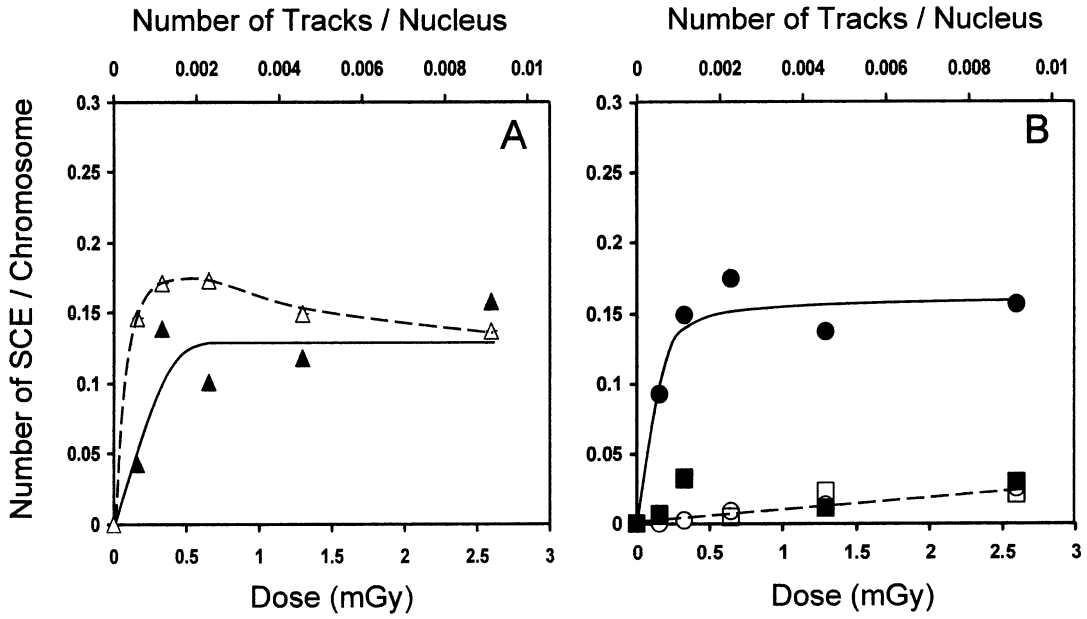

FIG. 2. Dose–response curves for induction of SCEs (total SCEs − background SCEs) by low-dose α-particle irradiation. Panel A: Wild-type CHO (▲) and DNA-Pkcs-deficient V3/vec cells (△) irradiated in G_1 phase. Panel B: Wild-type V79 (●) and RAD51C-deficient *irs3* cells irradiated in G_1 phase (0 h, ○), mid-S phase (8 h after G_1 release, ■), and late S/G_2 (13 h after G_1 release, (□).

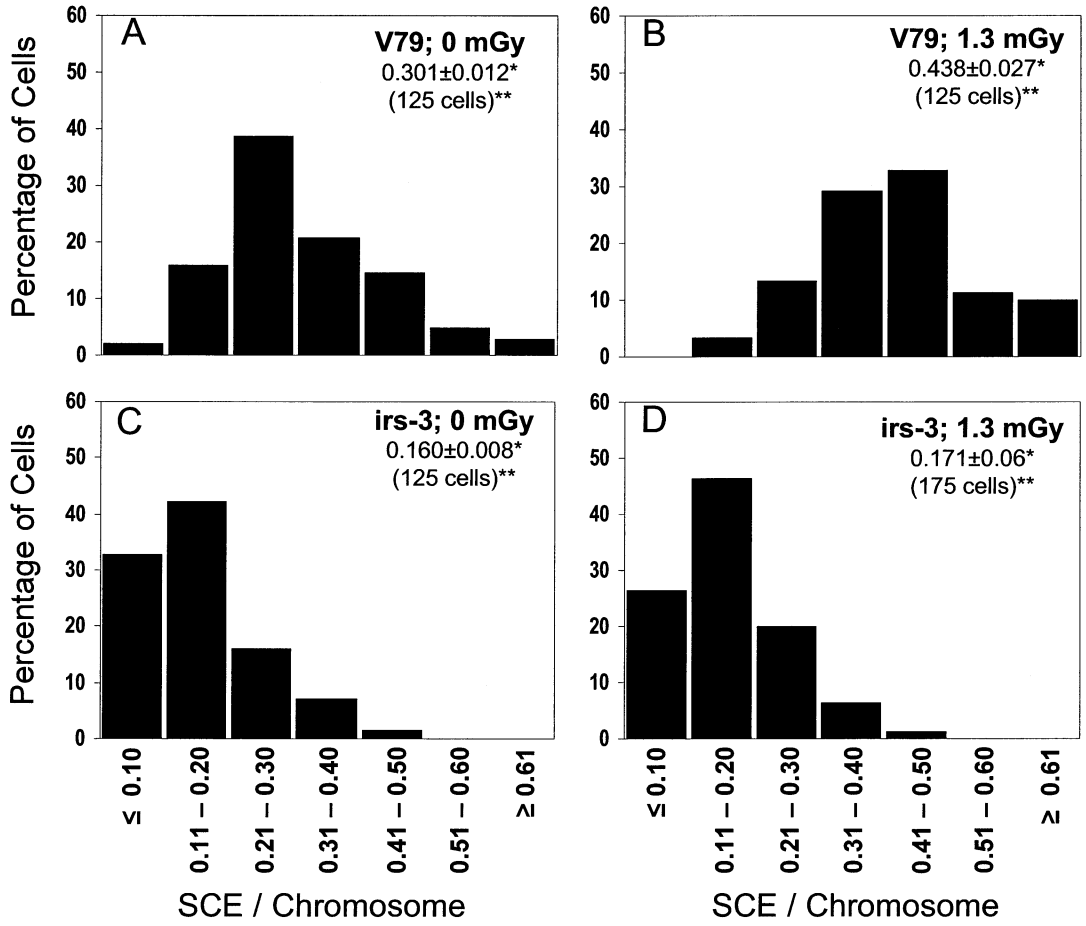

FIG. 3. Frequency distributions of SCE per chromosome for V79 (panel A: 0 mGy, panel B: 1.3 mGy) and *irs3* (panel C: 0 mGy, panel D: 1.3 mGy) cells. Values for mean SCE per chromosome ± 1 SEM (indicated by *) are reported for pooled data sets from several experiments (** indicates total number of cells scored).

doses for these experiments ranged from 0 to 2.6 mGy, corresponding to less than 1% of the cells experiencing a nuclear traversal by these α particles for the highest doses. The spontaneous levels of SCE per chromosome for unirradiated cells were approximately 0.34 for CHO and V79 cells, 0.40 for V3/vec cells, and only 0.16 for the HR-defective *irs3* cells.

The induced levels of SCEs in wild-type CHO cells rose rapidly for the lower doses, reaching a plateau at approximately 1.3 mGy, where on average only one cell in 217 experienced a nuclear traversal. This dose–response curve is virtually identical to those reported earlier in two independent studies (*5, 25*). There appeared to be a much greater bystander effect in NHEJ-defective V3/vec cells (Fig. 2A), with the maximal level of induced SCEs occurring for a mean dose of 0.35 mGy. This dose is about one-quarter the dose for the same effect in wild-type CHO cells, a relative sensitivity similar to the ratio of doses for equal cell killing of these cells. The induction of SCEs for identical mean doses of α particles in wild-type V79 and HR-defective *irs3* cells is shown in Fig. 2B. No significant induction of SCE in the *irs3* cells was observed after α-particle irradiation at 0, 8 or 13 h after release from G_1, when the cells had reached late S/G_2 phase.

More detailed analyses of the results reported in Fig. 2B are shown in Fig. 3A–D, in which the distributions of frequencies of both spontaneous SCEs per chromosome (0 mGy) and total (spontaneous plus induced) SCEs per chromosome after irradiation with 1.3 mGy in V79 and *irs3* cells are plotted. The frequency distributions of SCEs per chromosome on a per cell basis were approximately Poisson. Thus not only did the RAD51C-deficient *irs3* cells have significantly lower spontaneous SCE levels, but α particles did not significantly induce SCEs in HR-defective bystander cells, at least in this dose range. As was shown previously for CHO cells (*5, 25*), the steep increase in mean SCE frequencies after low-dose α-particle irradiation in wild-type V79 cells is the result of SCEs occurring in bystander cells; less than 1% of the cells would be traversed by an α particle after a dose of 1.3 mGy. On the other hand, no significant shift in mean SCE frequencies occurred in *irs3* cells (Fig. 3C, D); the mean SCE per chromosome was 0.160 ± 0.008 (SEM) for unirradiated cells and 0.171 ± 0.006 (SEM) after a mean dose of 1.3 mGy.

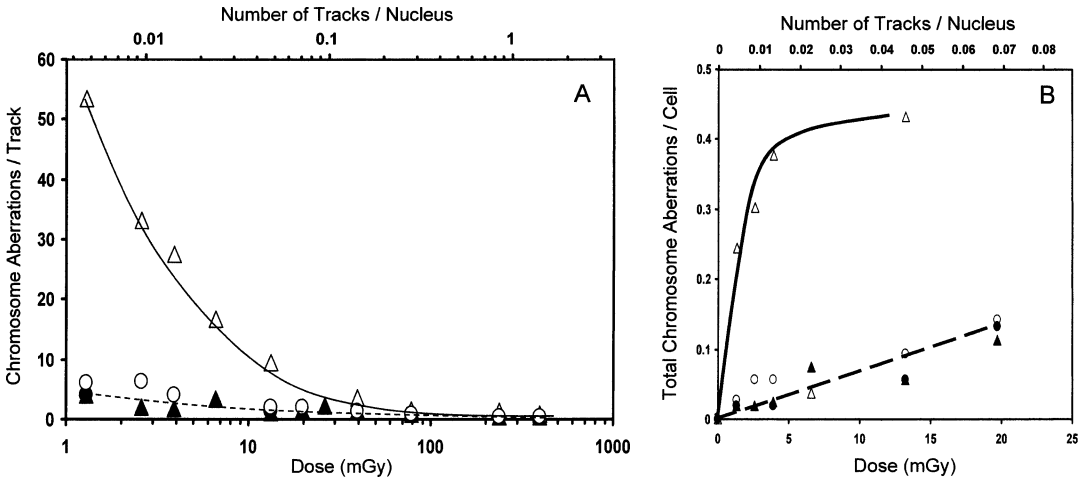

FIG. 4. Panel A. Dose–response curves for induction of chromosomal aberrations per α-particle track in the low-dose region up to 400 mGy, where a relatively large contribution to the effect derives from bystander cells. The effect per track is plotted as a function of the mean dose (lower abscissa) as well as the number of track traversals per nucleus (upper abscissa) for CHO (▲), V3/vec (△), V79 (●) and *irs3* (○) cells. Panel B: Dose–response curves for induction of chromosomal aberrations per cell by low-dose α-particle irradiation as a function of number of track traversals per nucleus (upper abscissa) and mean dose in mGy (lower abscissa) for G_1-phase CHO (▲), V3/vec (△), V79 (●) and *irs3* (○) cells treated with isoleucine-deficient medium.

We have previously shown a greatly enhanced bystander effect for chromosomal aberrations in cells defective in the NHEJ pathway (*7*). In that study, cells of a mouse DNA-PKcs knockout cell line irradiated with a mean dose of 1.7 mGy were shown to have a 10-fold higher number of chromosomal aberrations per α-particle track than wild-type cells, reflecting a 10-fold increase in the fraction of cells with chromosomal aberrations. As can be seen in Fig. 4A, a very similar result was found in the present study for the V3/vec hamster cell line deficient in DNA-PKcs. On the other hand, *irs3* cells demonstrate a dose–response curve for the induction of chromosomal aberrations that is identical to that of wild-type V79 cells, both for very low doses (Fig. 4B) and for doses up to 400 mGy when each cell received multiple hits (data not shown). It should be noted that in all these low-dose experiments, chromatid-type aberrations predominated, consistent with the hypothesis that the majority of DNA damage generated in bystander cells is single-stranded (e.g. base/sugar damage, single-strand breaks), not double-stranded (i.e. damage that would generate chromosome-type aberrations).

DISCUSSION

The data presented in Fig. 4 confirm our previous findings (*7, 9*) and indicate that bystander cells deficient in the NHEJ pathway are highly sensitive to the induction of chromosomal aberrations. High concentrations of reactive oxygen species have been reported in bystander cells (*10, 26*). We have hypothesized previously that complex DSBs resulting from the processing of single-strand breaks and closely opposed oxidative lesions, in addition to those occurring spontaneously, are efficiently repaired in wild-type cells, whereas they remained unrejoined or misrejoined in NHEJ-deficient cells (*7*). Such lesions would be highly clastogenic compared with oxidative base damage, which is presumably the major DNA lesion in wild-type bystander cells. This is consistent with the observation that mutations in wild-type bystander cells are almost entirely point mutations, whereas deletions are the predominant lesion in NHEJ-deficient cells (*8, 9*). Since chromosome-type aberrations are produced in mitosis after irradiation in G_1 phase, such complex DSBs must also be induced in G_1. The fact that the majority of aberrations induced in wild-type bystander cells were chromatid types also suggests that base damage is a principal lesion underlying the observed effects (*27*).

This hypothesis would presume that unrepaired and/or misrepaired DNA damage occurring in NHEJ-deficient bystander cells is not adequately repaired by the HR pathway. This conclusion is consistent with the observation that the bystander response for chromosomal aberrations in cells defective in the error-free HR pathway irradiated 0–13 h after subculture was small and identical to that of wild-type cells (Fig. 4). It is furthermore consistent with the radiosensitivity of these lines (Table 1); that is, the marked bystander response for chromosomal aberrations in the highly radiosensitive NHEJ-defective line compared to the very small bystander effect for wild-type and HR-deficient cells, both of which showed normal radiosensitivity.

These conclusions are also supported by the findings for SCE induction. A marked bystander effect for SCE induction was observed for both wild-type cell lines, whereas a much greater sensitivity to the induction of SCEs in by-

stander cells was apparent in the NHEJ-defective line as manifested by the steep rise in SCEs at very low doses (Fig. 2A). It has been postulated that exchanges between sister chromatids occur as one possible outcome of a homologous recombination process involving the formation of double Holliday junctions designed to correct errors present in the DNA prior to cell division (*14*). The enhanced bystander response for SCE induction could imply that an enhanced number of DNA lesions were present in NHEJ-deficient cells as they progressed into late S and G_2, stimulating an increased level of homologous recombination between sister chromatids in an attempt to repair these lesions. One could further surmise that this repair was not very successful, as reflected by the enhanced bystander response for chromosomal aberrations. Since all accounts to date suggest that SCEs arise through one possible outcome (crossover) of the homologous recombination process, the greatly diminished bystander response for the *RAD51C*-deficient *irs3* cells and the low frequency of spontaneous SCEs are consistent with their inability to carry out homologous recombination. To further address this issue, additional hamster cell lines mutated in other key HR proteins are currently being investigated

Regarding the above interpretation suggesting base damage as a critical lesion underlying SCE induction in bystander cells, it is also noteworthy that large increases in spontaneous SCEs have been reported in cells from patients with Bloom's syndrome. The very high SCE levels for Bloom's syndrome cells have been attributed to a defect in BLM-TOPO IIIa that is responsible for resolution of double Holliday junctions arising from base damage repair. As already mentioned, without an intact HR pathway, no SCEs would be expected. While interpretations at the level of molecular mechanisms are always attractive, it is also worth noting that branch migrations of double Holliday junctions over tens of Mb along the chromosome would be necessary to explain the sizes of the SCEs seen at the cytogenetic level.

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