Sequestration of Mammalian Rad51-Recombination Protein into Micronuclei

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Abstract. The mammalian Rad51 protein is involved in homologous recombination and in DNA damage repair. Its nuclear distribution after DNA damage is highly dynamic, and distinct foci of Rad51 protein, distributed throughout the nuclear volume, are induced within a few hours after γ irradiation; these foci then coalesce into larger clusters. Rad51-positive cells do not undergo DNA replication. Rad51 foci colocalize with both replication protein A and sites of unscheduled DNA repair synthesis and may represent a nuclear domain for recombinational DNA repair. By 24 h postirradiation, most foci are sequestered into micronuclei or assembled into Rad51-coated DNA fibers. These micronuclei and DNA fibers display genome fragmentation typical of apoptotic cell death. Other repair proteins, such as Rad52 and Gadd45, are not eliminated from the nucleus. DNA double strand breaks in repair-deficient cells or induced by the clastogen etoposide are also accompanied by the sequestering of Rad51 protein before cell death. The spindle poison colcemid causes cell cycle arrest and Rad51-foci formation without directly damaging DNA. Collectively, these observations suggest that mammalian Rad51 protein associates with damaged DNA and/or with DNA that is temporarily or irreversibly unable to replicate and these foci may subsequently be eliminated from the nucleus.

Key words: DNA repair • immunofluorescence • micronuclei • Rad51 • repairosome

Saccharomyces cerevisiae mutant screens have identified a large number of genes involved in DNA recombination and repair belonging to three major epistasis groups, nucleotide excision repair, error-prone repair, and double strand break (DSB)[1] repair (Friedberg et al., 1991; Petes et al., 1991). The Rad52-epistasis group, including Rad51, Rad52, and Rad54, represents a pathway for DSB repair by homologous recombination. Inactivation of Rad51, a structural and functional homologue of *Escherichia coli* RecA recombinase, renders yeast highly sensitive to ionizing radiation and dramatically reduces recombination frequencies (Shinohara et al., 1992; Heyer, 1994). Mammalian *Rad51* genes were identified on the basis of sequence similarity. They are transcribed at high levels in thymus, spleen, intestine, ovary, and testis and at lower levels in most other tissues (Morita et al., 1993; Shinohara et al., 1993). In cultured human cells, the HsRad51 protein is detected in multiple discrete foci in the nucleoplasm of a few cells by immunofluorescent antibodies. After DNA damage the percentage of cells with focally concentrated Rad51 protein increases in a time- and dose-dependent manner (Haaf et al., 1995). Chinese hamster ovary cells overexpressing CgRad51 show increased levels of homologous recombination and are more resistant to ionizing radiation (Vispé et al., 1998), whereas Rad51-deficient chicken lymphocytes in which a human Rad51 transgene is inactivated show increases in chromosome breaks and cell death (Sonoda et al., 1998). Thus, accumulating experimental evidence suggests that Rad51-mediated homologous recombination may also play an essential role in DNA repair of mammalian cells (Liang et al., 1998). Both yeast and human Rad51 appear to function as part of a larger recombination complex that includes Rad52 and other members of the Rad52-epistasis group (Shinohara and Ogawa, 1995; Benson et al., 1998).

Micronuclei (MN) originate from chromosomal material that is not incorporated into daughter nuclei during cell division. Different chemicals and cell treatments in-

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1. *Abbreviations used in this paper:* BrdU, 5-bromodeoxyuridine; ^{60}Co, cobalt 60; ^{137}Cs, cesium 137; DAPI, 4′,6-diamidino-2-phenylindole; DSB, double strand break; FISEL, fluorescence in situ end labeling; MN, micronuclei; RNAPII, RNA polymerase II; RPA, replication protein A; ss, single strand; XP, Xerodema pigmentosum.

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duce different MN types. MN caused by ionizing radiation or clastogens mostly contain acentric chromosome fragments. In contrast, MN induced by aneuploidogens contain whole chromosomes and stain positively for the presence of centromeres (Marrazzini et al., 1994; Verhaegen and Vral, 1994; Stopper et al., 1995). Determination of MN frequencies has been widely used as a dosimeter of human exposure to radiation or clastogenic and aneugenic chemicals, and for the detection and risk assessment of environmental mutagens and carcinogens (Heddle et al., 1991; Norppa et al., 1993; Hahnfeldt and Hlatky, 1994). In spite of the utility of the MN test as an in situ monitor of cytogenetic effects, our understanding of the connection between initial DNA damage and the formation of MN is still poor.

Here we show that in irreversibly damaged cells virtually all the Rad51 protein is sequestered, together with the irreparable DNA and the single strand (ss) DNA-binding replication protein A (RPA), into MN that undergo apoptotic genome fragmentation. The possible functional role of Rad51 foci in cells containing nonreplicating and/or irreparable DNA is discussed.

Materials and Methods

Cell Culture

The sources of the cell lines are as follows. Transformed rat TGR-1 fibroblasts (Prouty et al., 1993), J. Sedivy (Brown University, Providence, RI); mouse 3T3-Swiss cells, American Type Culture Collection (ATCC); adenovirus-transformed human 293 kidney cells, ATCC; human teratoma cells, B. King (Yale University, New Haven, CT); SV-40–transformed human LNL8 fibroblasts, S. Meyn (Yale University); SV-40–transformed fibroblasts from patients with Xeroderma pigmentosum group A (XPA) and F (XPF), P. Glazer (Yale University). PPL and KRA are primary human fibroblast strains, MYO are MyoD-transformed fibroblasts.

Monolayer cultures were grown in DME medium supplemented with 10% fetal bovine serum and antibiotics. Since XP cells are sensitive to daylight and luminescence from most lamps, they were grown in flasks which were wrapped with tin foil. The cells were detached from culture flasks by gentle trypsinization, pelleted, and then resuspended in phosphate-buffered saline (PBS; 136 mM NaCl, 2 mM KCl, 10.6 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3) prewarmed at 37°C.

To induce DSBs in DNA and recombinational repair, cell cultures were exposed to a cesium (^{137}Cs) or cobalt (^{60}Co) irradiator at doses of 1–10 Gy or to an ultraviolet (UVC) irradiator at doses of 10 J/m^2 and then allowed to recover for various time spans. Incubation of cells with the spindle poison colcemid (0.1 μg/ml for 24 h) resulted in the formation of multinuclei and MN containing entire chromosomes. Colcemid does not damage chromosomes directly. In addition, treatment with 2 μM etoposide, a drug that inhibits DNA topoisomerase II, was used for inducing DSBs and apoptosis in cells (Kaufmann, 1989; Mizumoto et al., 1994).

Antibody Probes

HsRad51 protein, expressed in *E. coli*, was isolated and used for preparation of rabbit polyclonal antibodies (Haaf et al., 1995). Western blotting experiments revealed that rabbit antiserum does not react significantly with any other proteins in mammalian cells except Rad51 and DMC1. Since DMC1 is not detectable in the cell substrates studied here using DMC1-specific antibodies (our unpublished results), this rabbit antiserum can be used as an immunofluorescent probe to visualize Rad51 in somatic cells. HsRad52 protein fused to a tag epitope of six histidines at the NH_2-terminal end was purified by metal chelate affinity chromatography. Since the resulting enzyme preparation contained some minor contaminating proteins that were well separated from Rad52, the protein was purified further on a preparative polyacrylamide gel. The band corresponding to Rad52 was cut out and the purified protein used for making antibodies in the rat. Mouse monoclonal antibody 30T14 recognizes Gadd45, a ubiquitously expressed mammalian protein that is induced by DNA damage (Smith et al., 1994). Monoclonal antibodies H4 and H14 bind specifically to the large subunit of RNA polymerase II (RNAPII) (Bregman et al., 1995). Anti-RPA antibody was provided by E. Gibbs (Memorial Sloan Kettering Cancer Center, New York, NY). Monoclonal antibody Pab246 against amino acids 88–93 of mouse p53 was purchased from Santa Cruz. Monoclonal antibody against 5-bromodeoxyuridine (BrdU) was obtained from Boehringer Mannheim. CREST autoimmune serum against centromeric proteins was used to label centromere dots during interphase (Haaf and Schmid, 1989).

Immunofluorescent Staining

Harvested cells were washed and resuspended in PBS. Cell density was adjusted to ∼10^6 cells/ml. 0.5-ml aliquots of this cell suspension were centrifuged onto clean glass slides at 800 rpm for 4 min, using a Shandon Cytospin. Immediately after cytocentrifugation, the preparations were fixed in absolute methanol for 30 min at −20°C and then rinsed in ice-cold acetone for a few seconds. After three washes with PBS, the preparations were incubated at 37°C with rabbit anti-HsRad51 antiserum, diluted 1:100 with PBS, in a humidified incubator for 30 min. For some experiments, the slides were simultaneously labeled with rat anti-HsRad52 antiserum, mouse monoclonal antibody to Gadd45, RPA, p53, RNAPII, or BrdU, or human CREST antiserum. The slides were then washed in PBS another three times for 10 min each and incubated for 30 min with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Dianova), appropriately diluted with PBS. Rad52, Gadd45, RPA, p53, RNAPII, BrdU incorporation, and centromeric proteins were detected with Cy3-conjugated anti-rat, anti-mouse, or anti-human IgG (Dianova). After three further washes with PBS, the preparations were counterstained with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) in 2× SSC for 5 min. The slides were mounted in 90% glycerol, 0.1 M Tris-HCl, pH 8.0, and 2.3% 1,4-diazobicyclo-2,2,2-octane.

Fluorescence In Situ End Labeling

Fluorescence in situ end labeling (FISEL) detects apoptotic cell death in situ by quantitating DNA strand breaks in individual nuclei. It uses terminal deoxynucleotidyl transferase to label the 3′-ends in fragmented genomic DNA with biotinylated nucleotide. 100 μl of reaction mix contain 1 μl (25 U) terminal transferase (Boehringer Mannheim), 20 μl 5× cacodylate buffer (supplied with the enyzme), 1 μl 0.5 mM biotin 16-dUTP, 3 μl 0.5 mM dTTP, and 75 μl double-distilled H_2O. Cytological preparations were incubated at 37°C for 1 h with this reaction mix. Washing the slides for three times for 5 min in PBS is sufficient to terminate the reaction. The incorporated biotin-dUTP is detected with Cy3-conjugated avidin.

In Situ Labeling of DNA Replication and DNA Repair Synthesis

The base analogue BrdU (10 μg/ml in the culture medium) was incorporated for 30 h in place of thymidine into the DNA of replicating cells. After Rad51-protein staining, the preparations were again fixed in a 3:1 mixture of methanol/acetic acid for several hours at −20°C. Since the anti-BrdU antibody only recognizes BrdU incorporated into chromosomal DNA if the DNA is in the single strand form, the slides were denatured in 70% formamide, 2× SSC for 1 min at 80°C and then dehydrated in an alcohol series. DNA replication synthesis was visualized by indirect anti-BrdU antibody staining. Only cells displaying a more or less uniform BrdU labeling of the entire nucleus were scored as replicating, which is consistent with the fact that the entire nuclear DNA is substituted with BrdU.

To detect cell cycle arrest and DNA repair synthesis after DNA damage, subconfluent fibroblast cultures were ^{60}Co irradiated with doses of 1 Gy and 10 Gy or with UVC with a dose of 10 J/m^2 and then cultured for various time spans. BrdU was added to the culture medium 2 h before cell harvesting. At the end of the BrdU pulse, slides were stained with anti-Rad51 and anti-BrdU antibodies, as described above. Cells undergoing unscheduled DNA repair synthesis (during the last 2 h of culture) were characterized by the appearance of only a few discrete BrdU foci, whereas replicating cells typically showed very many foci scattered throughout the entire nucleus.

Digital Imaging Microscopy

Images were taken with a Zeiss epifluorescence microscope equipped

Table I. Induction of Rad51 Foci in TGR-1 Cells after Irradiation with 10 Gy of ^{137}Cs and Their Elimination into Micronuclei

Time postirradiation	Percent of cells without foci	Percent of cells with type I* foci		Percent of cells with type II* foci	
		Nuclei	MN	Nuclei	MN
No treatment	93	6	0	1	0
3 h	80	8	<1	11	<1
16 h	73	9	8	1	9
30 h	72	1	13	1	13
4 d	90	0	4	0	6

*Type I nuclei and MN show weak to medium Rad51 immunofluorescence, whereas type II cells show strongly fluorescing foci. 1,000 cells were analyzed for each experiment.

with a thermoelectronically cooled charge-coupled device camera (model CH250; Photometrics), which was controlled by a Macintosh computer (Apple). Grayscale source images were captured separately with filter sets for FITC, Cy3, and DAPI. Grayscale images were pseudocolored and merged using Oncor Image (Oncor) and Adobe Photoshop software (Adobe Systems). It is worth emphasizing that although a digital imaging system was used, the immunofluorescent signals described here were clearly visible by eye through the microscope.

Results

Dynamic Nuclear Distribution of Rad51 Protein after DNA Damage

Previously we showed that the Rad51 protein is concentrated in multiple discrete foci in the nucleoplasm of cultured mammalian cells although in only a small percentage of cells. However, after DNA damage by γ irradiation, ultraviolet irradiation, or methyl methanesulfonate treatment, the number of cells with focally concentrated Rad51 protein increased considerably (Haaf et al., 1995). Since Western blot analysis did not detect a net increase in Rad51 protein in irradiated cells, it was concluded that DNA damage mainly affects the nuclear distribution of Rad51. Here we have analyzed the nuclear patterns of Rad51-protein foci in rat TGR-1 fibroblasts that were irradiated with a dose of 10 Gy of ^{137}Cs, which kills ~70% of cells. The percentage of cells with cytologically detectable Rad51-protein foci started to increase after 3 h (Table I). Rad51-positive nuclei contained up to several dozen discrete foci throughout their nucleoplasm. Many Rad51 foci had a double dot appearance. Immunofluorescence staining was largely excluded from the cytoplasm (Fig. 1 a).

When irradiated cells were then cultured for various times to allow DNA repair or apoptosis to occur, changes in the distribution of Rad51-protein foci were detected. Nuclear foci coalesced into larger clusters with extremely bright immunofluorescence intensity after 16–24 h, leaving only a few discrete foci or none in the nucleoplasm. In many irradiated cells we noticed parallel strings of Rad51 foci, typical of somatically paired higher-order structures (Fig. 1 b). 1 or 2 d after γ irradiation, these large Rad51 clusters showed a highly nonrandom localization near the nuclear periphery (Fig. 1 c). Subsequently, the Rad51 clusters were excluded into MN. The nucleoplasm of the original nucleus was virtually cleared of Rad51-protein foci and Rad51 immunofluorescence was detected only in MN (Fig. 1 d; Table I). The highest number of MN (approximately three per cell) as well as the highest number of Rad51-positive MN (approximately 30%) were observed 16 h after irradiation. 2 d postirradiation, the majority of MN dis-

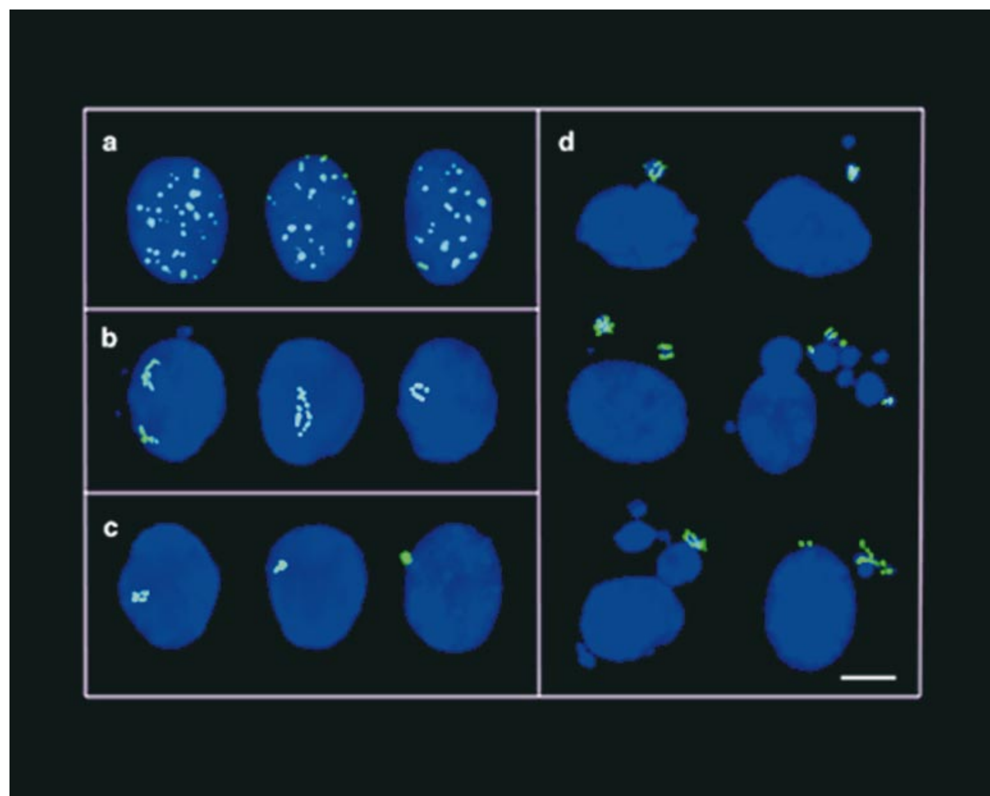


Figure 1. Dynamic changes in the higher-order nuclear organization of Rad51 foci after DNA damage. TGR-1 fibroblasts were irradiated with 10 Gy of ^{137}Cs and then allowed to recover for various times. Rad51 protein is stained by green immunofluorescence, nuclei are counterstained with DAPI. 3 h after irradiation (a), Rad51 foci are distributed throughout the entire nuclear volume. After 16 h (b), clusters and linear strings of Rad51 foci are formed. After 30 h (c), Rad51 clusters move towards the nuclear periphery and are eliminated into MN. 2 d after γ irradiation (d), all Rad51 foci are excluded into MN and the nucleus is cleared of detectable Rad51-protein foci. Note the double dot appearance of Rad51 foci (a) and the paired higher-order structures (b–d). Bar, 10 μm.

played apoptotic genome fragmentation, as determined by FISEL (data not shown).

In this context, it is important to emphasize that analysis of a certain time postirradiation provides a temporary snapshot of DNA repair and apoptotic processes in a heterogenous cell population. Moreover, even in a synchronized cell culture apoptosis is a highly asynchronous process occurring over a 2- or 3-d period after the same stimulation (Messam and Pittman, 1998). This may explain why the predominant nuclear patterns are changing over time but not all cells show identical Rad51 staining.

Rad51 Foci in Repair-deficient Cells and in Spontaneously Occurring MN

In addition to irradiated normal fibroblasts, we have analyzed cells of a patient suffering from the recessive hereditary disorder Xeroderma pigmentosum group A (XPA). XPA complementing protein is responsible for lesion recognition by the nucleotide excision repair system (Jones and Wood, 1993). We reasoned that unexcised lesions can lead to increased levels of DSBs, even without exposure to DNA-damaging agents. As expected, untreated XPA cultures displayed the same nuclear staining patterns, i.e. randomly distributed Rad51 foci, large clusters of Rad51 foci, and Rad51-positive MN, as irradiated TGR-1 cells (data not shown). This strongly argues in favor of the notion that Rad51 foci are induced by DNA damage.

Rapidly dividing immortalized and tumor cell lines always exhibit a baseline frequency of MN even without exposure to DNA-damaging agents. In SV-40–transformed human fibroblasts, breast cancer and teratoma cells, and mouse 3T3-Swiss and rat TGR-1 fibroblasts, 10–30% of these spontaneously occurring MN exhibited Rad51-protein foci (data not shown). The question whether Rad51-positive and Rad51-negative MN are qualitatively different remains to be answered.

Induction of Rad51-Protein Foci by Clastogens and Aneuploidogens

Topoisomerase II binds covalently to double strand DNA,

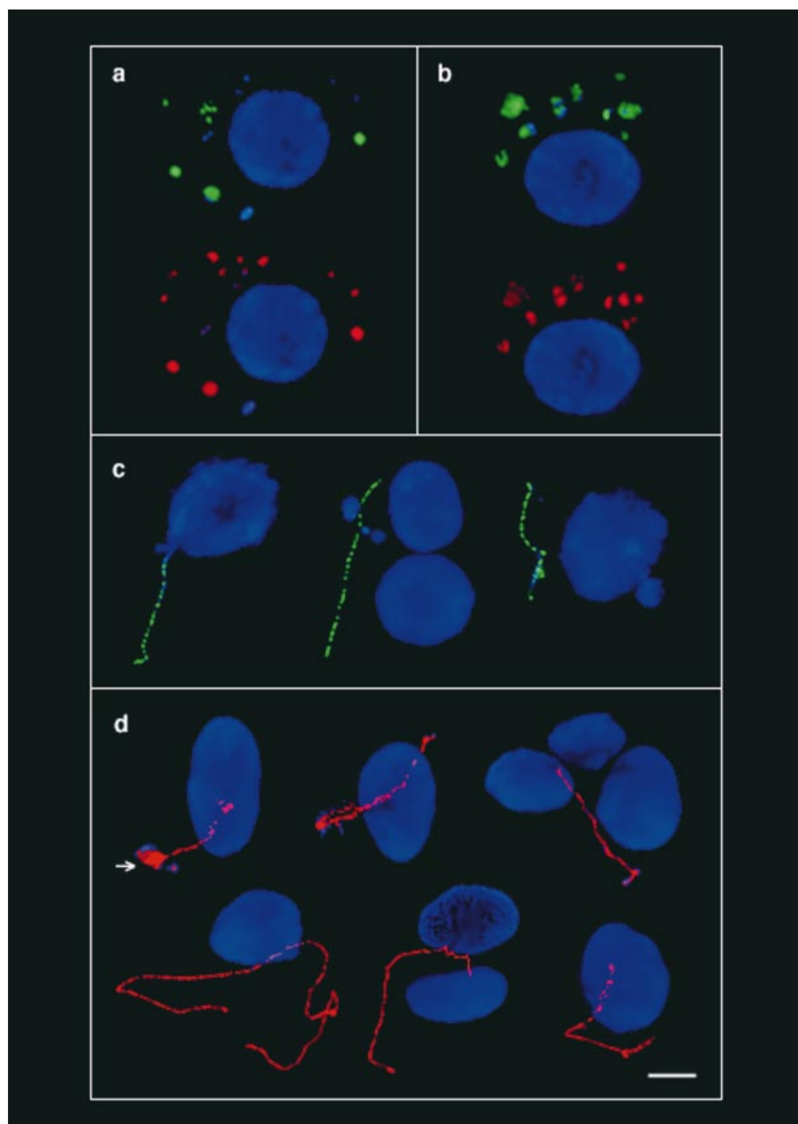


Figure 2. Genome fragmentation in Rad51-positive MN and DNA fibers. (a and b) MN, 2 d after treatment of TGR-1 cultures with etoposide (a) and colcemid (b), contain Rad51 protein and fragmented DNA. Top nuclei, green anti-Rad51 antibody; bottom nuclei, red FISEL staining of the same cell. Probably due to the proteolytic cleavage of Rad51 during apoptosis, FISEL-positive MN display a dispersed Rad51 immunofluorescence. (c and d) DNA fibers extruded from TGR-1 nuclei, 2 d after γ irradiation. Chromatin is counterstained with DAPI. The Rad51 signals appear as beads on a string on the linear DNA molecule (c, green), whereas the remaining nuclei are devoid of detectable Rad51 staining. DNA strand breaks in chromatin fibers are detected by FISEL (d, red). Some fibers appear to condense into MN (arrow). Bar, 10 μm.

Table II. Induction of Rad51 Foci and Genome Fragmentation by Etoposide Treatment of TGR-1 Cells

Etoposide treatment	Percent of apoptotic cells*	Percent of cells without foci	Percent of cells with type I‡ foci		Percent of cells with type II‡ foci	
			Nuclei	MN	Nuclei	MN
No treatment	6	93	6	0	1	0
2 h	—	92	4	1	1	2
5 h	—	92	3	2	1	2
12 h	17	87	8	2	1	2
18 h	24	79	3	8	1	9
24 h	33	82	2	2	6	8
36 h	47	83	2	5	1	9

*Detected by FISEL.
‡Type I nuclei and MN show weak to medium Rad51 immunofluorescence, whereas type II cells show strongly fluorescing foci. 500 cells were analyzed for each experiment.

cleaves both strands, and then reseals the cleaved complex (Osheroff et al., 1991). Etoposide interferes with this breakage and reunion cycle, trapping the enzyme in the cleaved complex. The resulting DSBs lead to apoptotic cell death (Mizumoto et al., 1994; Kaufmann, 1989). After adding etoposide to the culture medium, the percentage of nuclei and MN with Rad51 foci steadily increased (Fig. 2 a, green; Table II). The induction of Rad51 foci and MN formation were accompanied by apoptotic genome fragmentation (Fig. 2 a, red). At 36 h, ~50% of the cells showed incorporation of fluorescent nucleotides by FISEL (Table II).

The mitotic spindle poison colcemid causes whole chromosomes to be excluded into MN without directly inducing DNA damage. Surprisingly, when colcemid-treated cells were allowed to recover for 24 h in drug-free medium, over 30% of the induced MN contained very brightly fluorescing Rad51 foci (Fig. 2 b, green; Table III). Most of these Rad51-positive MN, 24 h after colcemid, did not yet contain abundant DNA breaks detectable by FISEL. However, FISEL will not detect a low level of breaks, which are likely to be present in colcemid-arrested MN containing highly condensed nonreplicating chromosomes. When cells were grown for one or two more days in the absence of the drug, most MN stained FISEL positively and were apoptotic (Fig. 2 b, red; Table III). At the same time, the percentage of Rad51-containing MN decreased dramatically. In addition, the Rad51 protein was no longer concentrated in discrete foci but appeared to disperse throughout the entire MN volume. This is consistent with the fact that mammalian Rad51 is proteolytically cleaved during apoptosis (Flygare et al., 1998). Mitotic arrest after colcemid seems to trigger a cascade that induces the sequestering of Rad51 protein into MN and drives apoptotic events. In this light, apoptosis may be considered as a special form of aberrant mitosis (Ucker, 1991; Shi et al., 1994).

Table III. Rad51 Foci and Genome Fragmentation in Colcemid-induced Micronuclei of TGR-1 Cells

Treatment	Number of MN in 1,000 cells	Percent of nuclei showing*			
		Rad51−/ FISEL−	Rad51+/ FISEL−	Rad51−/ FISEL+	Rad51+/ FISEL+
No treatment	93	75	12	11	2
Colcemid‡	—	85	6	9	0
1 d after colcemid	1293	54	31	14	1
2 d after colcemid	1061	45	9	40	6
3 d after colcemid	769	43	7	46	4

*Cells with a fragmented genome show fluorescence in situ end labeling (FISEL+), whereas normal cells show absence of end labeling (FISEL−). Rad51+, cells with Rad51 foci; Rad51−, cells without foci.
‡TGR-1 cells were grown for 24 h in medium containing 0.1 μg/ml colcemid to induce MN formation. 18% of the colcemid-treated cells were arrested at metaphase, 17% showed multinuclei (>10 MN), and 65% had no MN. The cells were then allowed to recover for various times in the absence of the drug. 500 cells were analyzed for each experiment.

Taken together, our results suggest that induction of nuclear Rad51 foci and their elimination into MN is a fundamental process which occurs after exposure of cells to irradiation, clastogens, or aneuploidogens before cell death. Apoptotic genome fragmentation, as detected by FISEL, is a secondary event resulting from cellular responses to DNA damage and/or irreversible growth arrest.

Rad51-DNA Fibers Are Eliminated from the Nucleus

In a few (<1%) irradiated or drug-treated cells, we observed fiber-like Rad51 structures, up to 100 μm in length, that were eliminated from the nuclei. These fibers stained positively with DAPI and, hence, are likely to contain linear DNA molecules covered with Rad51 (Fig. 2 c, green). FISEL demonstrated that whole DNA fibers consist of fragmented DNA typical of apoptosis (Fig. 2 d, red). Since these linear DNA fibers contain numerous DSBs and/or single strand nicks, they are likely to be held together by Rad51 and other proteins. They appeared to be extruded through the nuclear membrane and condense into MN (Fig. 2 d, arrow). Previously we have shown that Rad51 foci formed in the nucleus after DNA damage are the same nuclear areas where Rad51 forms filaments on ssDNA (Raderschall, E., E.I. Golub, and T. Haaf, manuscript submitted for publication). Therefore, it is plausible to assume that the observed DNA fibers represent rather long Rad51 filaments that are eliminated from the nucleus, because they could not be repaired by homologous recombination.

Cell Cycle Arrest of Cells with Focally Concentrated Rad51-Protein

Simultaneous Rad51-protein immunofluorescence (Fig. 3 a, green) and anti-BrdU antibody staining (Fig. 3 a, red) demonstrated that nuclei with focally concentrated Rad51 protein do not undergo DNA replication synthesis. BrdU was incorporated into replicating DNA of unsynchronized, untreated cell cultures for 30 h. Virus-transformed human kidney cells (line 293) and fibroblasts (line LNL8), as well as fibroblast lines of patients with XPA and XPF repair defects exhibited detectable Rad51 foci in a percentage of nuclei even without induced DNA damage. In each experiment, 100 nuclei with prominent Rad51 foci and 100 nuclei without detectable Rad51 foci were analyzed after staining with fluorescent anti-BrdU antibody. Only cells with intense BrdU labeling of the entire nucleus were scored as cycling cells (Fig. 3, a and b, red). The

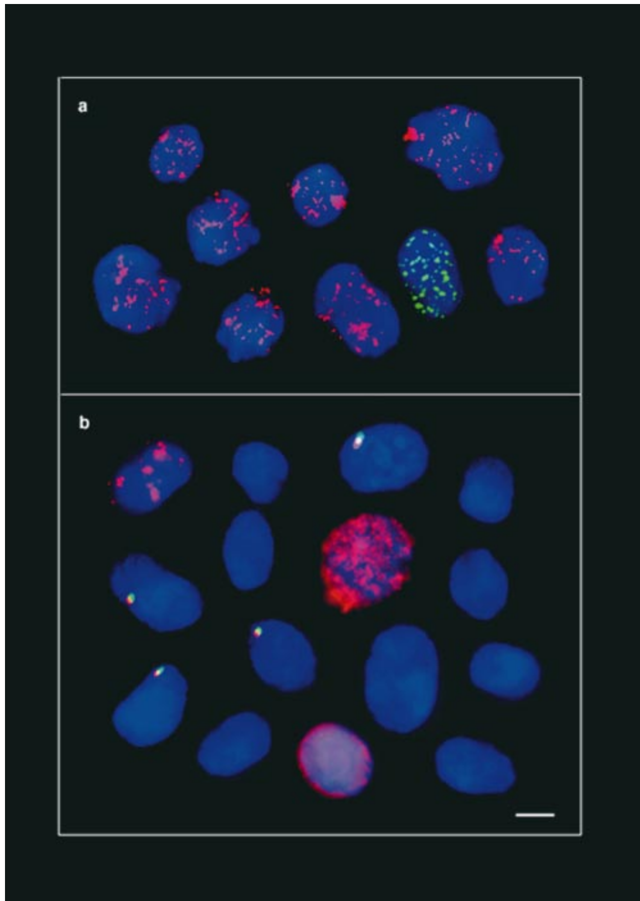


Figure 3. Cell cycle arrest and DNA repair synthesis of Rad51-foci–positive cells. (a) Simultaneous staining of Rad51 protein (green) and replicating DNA (red) in an exponentionally growing, untreated XPA fibroblast culture. BrdU was incorporated into DNA for 30 h and detected with red anti-BrdU antibody. Note that the Rad51-positive cell is devoid of BrdU label. (b) Colocalization of Rad51 foci (green) with DNA repair sites after DNA damage. Human PPL fibroblasts were irradiated with a dose of 1 Gy. BrdU was added 24 h postirradiation for 2 h. In contrast to replicating cells, which show strong overall BrdU staining, cells undergoing DNA repair synthesis exhibit only few discrete BrdU-incorporation sites (red). Bar, 10 μm.

BrdU-substituted DNA was located in discrete replication foci throughout the nucleus. In all cases, >90% of the cells with focally concentrated Rad51 protein were found to be BrdU-staining negative (Table IV). In contrast, 30–50% of the cells without Rad51 foci from the same cultures showed BrdU incorporation, indicative of cycling cells. This suggests that Rad51-foci–positive cells in an exponentially growing culture are arrested during the cell cycle.

In addition, we have analyzed the replication behavior of primary human PPL fibroblasts after γ irradiation with doses of 1 Gy and 10 Gy. BrdU was added to the cultures at 6, 24, and 48 h postirradiation for 2 h. As expected, the percentage of Rad51-positive nonreplicating cells rose (up to 50% of all cells of a culture), as time after irradiation and radiation dose increased (Fig. 4, solid black bars).

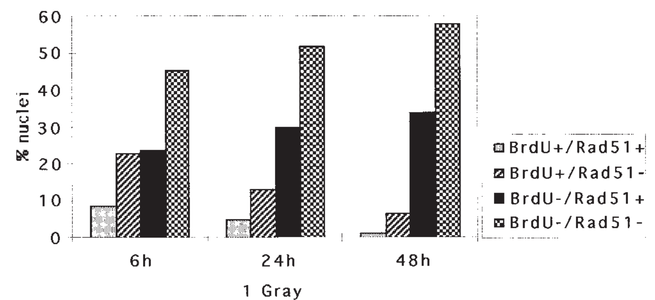
Table IV. Cell Cycle Arrest of Rad51-Foci–positive Cells without Induced DNA Damage

	Percent of Rad51-positive nuclei*		Percent of Rad51-negative nuclei*	
Cell line	BrdU−/ Rad51+	BrdU+/ Rad51+	BrdU−/ Rad51−	BrdU+/ Rad51−
293	97	3	53	47
LNL8	98	2	68	32
XPA	94	6	64	36
XPF	100	0	74	26

*Cells undergoing DNA replication during the last 30 h of culture show overall BrdU incorporation (BrdU+), whereas noncycling cells show absence of BrdU staining (BrdU−). 100 cells with nuclear Rad51 foci (Rad51+) and 100 cells without focally concentrated Rad51 protein (Rad51−) were screened for each cell line.

DNA Repair Synthesis Occurs at Rad51 Foci

After ^{60}Co or UV irradiation of PPL fibroblasts, the number of replicating cells decreased in a time- and dose-dependent manner. At the same time, cells undergoing DNA repair synthesis and the number of Rad51-foci–positive cells increased (Fig. 4; Table V). In contrast to replicating cells that displayed BrdU incorporation throughout their entire nuclear volume, cells undergoing DNA repair were endowed with only a few or even single BrdU foci (Fig. 3 b, red). After combined staining of Rad51 and BrdU incor-



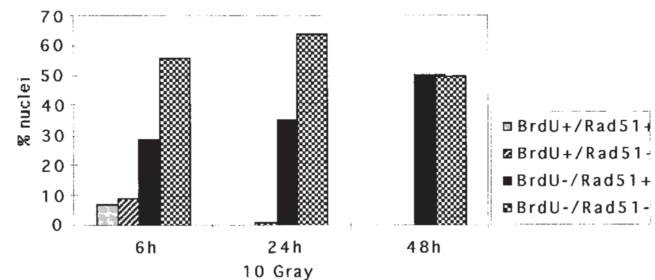


Figure 4. Frequencies of Rad51-foci–positive nonreplicating cells (solid black bars) after γ irradiation. Human PPL fibroblasts were irradiated with doses of 1 Gy and 10 Gy and analyzed at different times postirradiation. Cells undergoing DNA replication at a specific time point were scored as BrdU+, whereas nonreplicating cells were BrdU−. Simultaneously, the cells were stained for the presence (Rad51+) or absence (Rad51−) of Rad51 foci. At least 300 cells were analyzed for each experiment.

Table V. Colocalization of Rad51 Foci and DNA Repair Sites

Fibroblast strain/ treatment	Percent of cells with DNA repair synthesis*			Percent of replicating cells*	
	Rad51+/Co+	Rad51+/Co−	Rad51−	Rad51+	Rad51−
PPL/6 h after 1 Gy of ^{60}Co	10	1	24	9	22
PPL/6 h after 10 Gy of ^{60}Co	11	2	24	7	9
PPL/24 h after 1 Gy of ^{60}Co	12	1	17	5	13
PPL/24 h after 10 Gy of ^{60}Co	22	0	0	0	1
PPL/24 h after 10 J/m^2 of UVC	17	2	5	5	9
KRA/24 h after 10 J/m^2 of UVC	16	3	4	6	17
MYO/24 h after 10 J/m^2 of UVC	15	0	6	1	13

*BrdU was added at 6 h or 24 h postirradiation for 2 h. Cells with only few discrete BrdU foci were assumed to undergo DNA repair synthesis, whereas nuclei with strong overall BrdU staining were considered replicating. Rad51+, cells with Rad51 foci; Rad51−, cells without foci. Co+, cells with colocalization of Rad51 foci and DNA repair sites; Co−, cells without colocalization.

poration, 10–20% of cells from irradiated cultures showed colocalization of Rad51 foci and DNA repair sites (Fig. 3 b; Table V). We conclude that DNA damage-induced Rad51 foci mark a subset of cells which have entered the DNA repair synthesis pathway. In this light, Rad51 foci may correspond to nuclear repair sites where Rad51-mediated homologous recombination takes place.

RPA but Not Rad52 Is Sequestered into MN

Studies in yeast (Shinohara et al., 1992; Milne and Weaver, 1993) and humans (Shen et al., 1996; Benson et al., 1998) have shown physical interaction between Rad51 and Rad52 proteins both in vitro and in vivo. Double immunofluorescence with rabbit anti-Rad51 and rat anti-Rad52 antibodies on γ-irradiated TGR-1 cells showed that both proteins are enriched in nuclear foci but that the frequency of Rad51-Rad52 colocalizations was relatively low (Fig. 5 a, left nucleus). The 5–10% of foci which were immunoreactive with both antibodies may show colocalization due to functional interactions between the two proteins. Mock experiments using rabbit anti-Rad51 and human anti-centromere protein (CREST) antibodies yielded up to 5% double-staining foci, which are due to chance associations between Rad51 foci and centromeres (data not shown). Notably, Rad52-protein foci (Fig. 5 a, red) remained in the nucleus throughout the entire time course, whereas Rad51 foci were segregated into MN (Fig. 5, a–c, green). Gadd45 (Fig. 5 b, red), an inducible DNA repair protein that is stimulated by p53 (Smith et al., 1994), was detected in 3–5% of Rad51 foci, which is in the range of chance associations. Gadd45 was also not eliminated into MN. Biochemical evidence suggests specific protein–protein associations between HsRad51 and p53 (Stürzbecher et al., 1996). However, anti-p53 antibody staining revealed that the Rad51 foci are not particularly enriched with p53 protein (data not shown). In addition, HsRad51 was reported to be associated with a RNAPII holoenzyme (Maldonado et al., 1996). Although RNAPII was immunolocalized in discrete nuclear foci, as reported previously (Bregman et al., 1995),

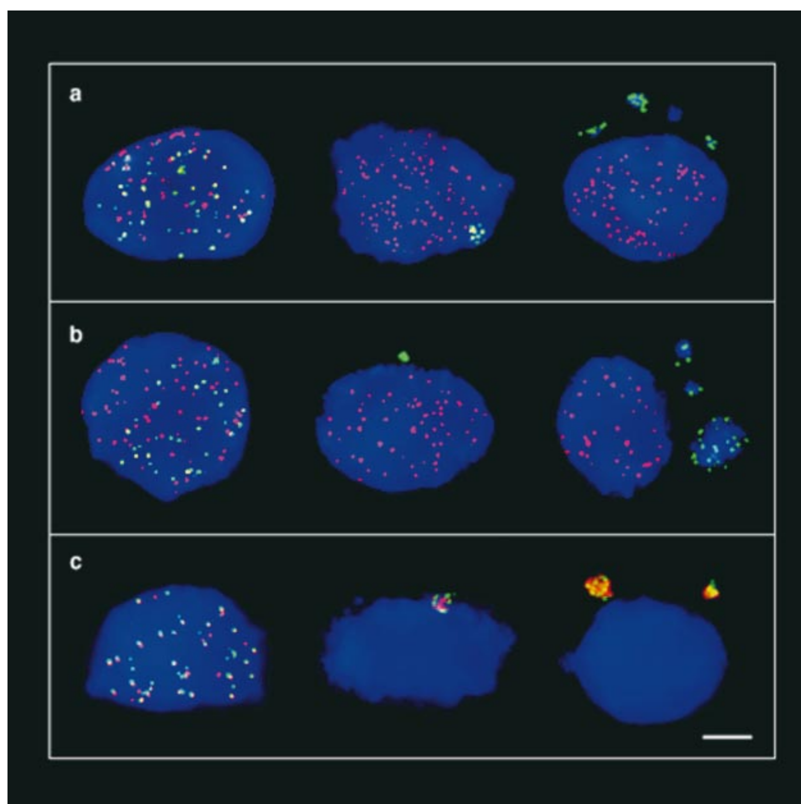


Figure 5. Spatial relationships between Rad51 foci and Rad52, Gadd45, and RPA after DNA damage. TGR-1 nuclei, 3 (left), 16 (middle), and 30 h (right) after γ irradiation. Rad51-protein foci show green immunofluorescence. Rad52 (a), Gadd45 (b), and RPA (c) are detected by red antibody probes. Nuclei are counterstained with DAPI. Only RPA colocalizes with Rad51 and segregates into MN. Bar, 7 μm.

these transcription complexes did not coincide with Rad51 foci (data not shown). One day after γ irradiation >50% of Rad51 foci are enriched with RPA (Golub et al., 1998). Here we show that 2 or 3 d after DNA damage, RPA foci (Fig. 5 c, red) are also excluded into MN. Both Rad51 and RPA bind to ssDNA and may be sequestered along with the damaged (ss)DNA into MN.

Discussion

Purified Rad51 proteins from yeast (Ogawa et al., 1993; Sung, 1994) and humans (Benson et al., 1994; Baumann et al., 1996; Gupta et al., 1997) are able to bind single and double strand DNA and catalyze strand-exchange reactions in vitro. The mouse *Rad51* cDNA can complement a *S. cerevisiae Rad51* mutation (Morita et al., 1993), suggesting a conserved function(s). In yeast, the Rad51 family also includes Rad55, Rad57, and DMC1, which has a meiosis-specific function (Game, 1993). The entire yeast genome has been sequenced and there are no other RecA-related genes in *S. cerevisiae*. Mammals have an expanded Rad51 family that contains at least nine RecA-related genes: HsRad51 (now also called HsRad51a), XRCC2 and XRCC3 (Liu et al., 1998), HsRad51b/hRec2 (Albala et al., 1997), HsRad51c (Dosanjh et al., 1998), HsRad51d (Pittman et al., 1998), R51H2 and R51H3 (Cartwright et al., 1998), and HsDMC1 (Habu et al., 1996). However, since the Rad51 is essential in mammalian cells (Lim and Hasty, 1996; Tsuzuki et al., 1996), no other RecA-related gene can replace its function. Whether DNA recombination/repair in mammalian cells requires more proteins than in yeast or whether there are multiple related pathways is currently unknown. Unlike HsRad51, none of the other Rad51-family members interacts with itself. Therefore, similar to yeast Rad55 and Rad57, they may serve as accessory proteins to stabilize or enhance the Rad51 activity, rather than as functional homologues of Rad51.

DSB repair is unlikely to be accomplished by Rad51 alone, but may involve protein–protein interactions with the Rad52-epistasis group and other proteins (Shinohara and Ogawa, 1995). Like in yeast, HsRad51 protein can interact with itself and with HsRad52 (Shen et al., 1996; Benson et al., 1998) and HsRad54 (Golub et al., 1997). In addition, it can be associated with numerous other proteins including the tumor suppressor p53 (Stürzbecher et al., 1996), the nonreceptor tyrosine kinase c-Abl (Yuan et al., 1998), the BRCA1 (Scully et al., 1997) and BRCA2 (Sharan et al., 1997) proteins which are mutated in inherited breast cancer, the ubiquitin-conjugating enzyme Ubc9 (Kovalenko et al., 1996), and the RNAPII-transcription complex (Maldonado et al., 1996). The biological significance of all these biochemical interactions remains unknown. Our experiments have shown that the number of Rad51-Rad52 colocalizations in the cell nucleus is only slightly higher than by chance and that the dynamic behavior of Rad51 and Rad52 foci after DNA damage are largely different. In particular, Rad52-protein foci are not extruded into MN along with the damaged DNA. We did not find any significant colocalization of Rad51 foci with Gadd45, p53, and RNAPII. These data could be interpreted to mean that mammalian Rad52, Gadd45, p53, and RNAPII have little or no function during recombinational repair. However, studies in yeast (Gasior et al., 1998) have indicated that Rad52 may act before Rad51 and is required for Rad51-foci formation during meiotic recombination. Therefore, other interpretations are also possible.

For example, Rad52 may simply facilitate formation of the Rad51 filament and then be displaced. Or, Rad51 and Rad52 may have very different stoichiometries of interaction with DNA. In this context, it is also important to note that immunofluorescent assays are strongly biased towards detection of large protein accumulations. Generation of a dot-like fluorescence signal in the interphase nucleus requires the binding of at least 100 fluorescent antibody molecules (Haaf et al., 1991). As a result, we may not be able to mark the majority of cells where DSBs are repaired by homologous recombination. Most Rad51 filaments which are formed as intermediates of homologous recombination may be too short to be seen by immunofluorescence staining. Rad51 foci that are observed 3–48 h postirradiation may contain unusually large Rad51 filaments which did not find a partner for homologous recombination and are irreparable. When a cell contains such a long filament, it stops DNA replication and, consequently, bright Rad51 foci are seen mainly in those cells which are arrested during the cell cycle or prone to die. Small Rad51 foci representing recombination intermediates disappear after completion of homologous recombination. In contrast, most detectable Rad51 foci containing large irreparable DNA segments finally coalesce and are removed into MN. Thus far, only the ssDNA-binding protein RPA was shown to colocalize with Rad51 in nuclear foci and MN after DNA damage. RPA facilitates homologous pairing and DNA–strand exchange, mediated by Rad51 (Baumann et al., 1996; Sugiyama et al., 1997; Gupta et al., 1998).

Various treatments that cause DNA damage increased the number of cells with Rad51-protein foci drastically. This increase was not connected with a significant induction of Rad51-protein synthesis (Haaf et al., 1995). Considering that Rad51 mediates homologous recombination in vitro, its relocalization as a result of DNA damage suggests a role for the protein foci in recombinational DNA repair. Homologous recombination starts with the 5′ to 3′ exonucleolytic degradation of one DNA strand, producing rather long (>1 kb) 3′-overhanging ssDNA tails (Osman and Subramani, 1998). This ssDNA associates with Rad51 protein, forming the ssDNA-Rad51 filament, which is considered to be the key element for promoting the pairing and strand exchange between ssDNA and homologous double strand DNA (Benson et al., 1994; Baumann et al., 1996; Gupta et al., 1997). Recently we have shown that Rad51 foci are located at ssDNA regions formed after DNA damage, indicating that these are the nuclear areas where Rad51 forms filaments on ssDNA tails (Raderschall, E., E.I. Golub, and T. Haaf, manuscript submitted for publication). The present observation that these foci also colocalize with RPA and sites of unscheduled DNA repair synthesis provides further evidence that Rad51-protein foci represent a repairosome-type assembly for recombinational DNA repair.

Several hours after their formation, the Rad51 foci started to coalesce. They moved towards the nuclear periphery and then out of the cells into MN, where the DNA

is subject to apoptotic degradation. The reasons for this relocalization are unknown. However, there are at least two competing hypotheses that could explain the dynamic behavior of Rad51 after DNA damage. One hypothesis is that Rad51 forms filaments on damaged or just unmasked DNA that later move into the MN but it does not mediate this process. Given that both Rad51 and RPA (but, thus far, no other repair proteins) are found in MN, this argues that MN contain broken chromosomes and/or ssDNA molecules which likely bind with these proteins. The other hypothesis is that Rad51 protein may participate, actively or passively, in detecting and removing irreversibly damaged, irreparable DNA from cells, followed by DNA degradation in MN. Although colcemid-treated cells are likely to have a very low level of breaks, they exhibit very prominent Rad51 foci. This supports the idea that Rad51 protein forms foci on nonreplicating DNA with just a single or very few DNA breaks and, if the DNA is not repaired, then this chromosome or chromosome fragment is excluded into MN. Rad51 may not only function in DSB repair but also be involved in nuclear events linked to DNA replication and cell cycle arrest. Knockout studies have already suggested that, apart from recombination/repair, mammalian Rad51 may perform a novel function(s), i.e., in cell proliferation. In contrast to the viability of *E. coli recA* and yeast *ScRad51* mutants, homozygous *MmRad51*$^{-/-}$ knockouts convey an embryonic lethal phenotype and cell lethality in mice (Lim and Hasty, 1996; Tsuzuki et al., 1996).

Studies on synchronized mouse cells have demonstrated that mammalian *Rad51* is preferentially expressed from G_1/S to G_2 phase (Yamamoto et al., 1996). In phytohemagglutinin-stimulated human lymphoblasts, Rad51 appeared to form nuclear foci during S phase (Tashiro et al., 1996). However, this does not explain the increase of Rad51-protein foci in cells after exposure to irradiation, clastogens, or aneuploidogens described here. Blood lymphocytes undergo terminal differentiation processes upon metabolic activation, which may involve homologous recombination events. In our experience, synchronized TGR-1 cultures (14 h after serum starvation) with $\sim$60% S phase cells (Prouty et al., 1993) and many other normal cell types tested (data not shown) display only very few (1% or less) nuclei with bright (type II) Rad51 foci. BrdU labeling of replicating cells in widely different substrates and irradiation experiments also demonstrated directly that most cells with bright Rad51 foci are arrested during the cell cycle.

Taken together, our data suggest that prominent Rad51-protein foci are formed by association of Rad51 protein with damaged DNA that has irreversibly or temporarily lost its ability to replicate and/or is irreparable. Rad51 may act as a "sensor" of DNA damage which arrests the cell cycle to allow DNA repair or cell death to occur. In the absence of repair, MN form and this may be, directly or indirectly, mediated by Rad51. On the other hand, at present we cannot exclude the possibility that Rad51 is just a passive passenger (i.e., without any function) on irreversibly damaged DNA. Stably transfected cells overexpressing HsRad51 may offer a convenient tissue culture model to answer this question and to study the newly acquired effects of mammalian Rad51 on cell proliferation and programmed cell death.

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