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Rad51-deficient vertebrate cells accumulate chromosomal breaks prior to cell death

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Yeast *rad51* mutants are viable, but extremely sensitive to γ-rays due to defective repair of double-strand breaks. In contrast, disruption of the murine *RAD51* homologue is lethal, indicating an essential role of Rad51 in vertebrate cells. We generated clones of the chicken B lymphocyte line DT40 carrying a human RAD51 transgene under the control of a repressible promoter and subsequently disrupted the endogenous *RAD51* loci. Upon inhibition of the RAD51 transgene, Rad51$^-$ cells accumulated in the G_2/M phase of the cell cycle before dying. Chromosome analysis revealed that most metaphase-arrested Rad51$^-$ cells carried isochromatid-type breaks. In conclusion, Rad51 fulfils an essential role in the repair of spontaneously occurring chromosome breaks in proliferating cells of higher eukaryotes.

Keywords: chromosome aberration/DT40/double-strand break/Rad51 disruption

Introduction

Studies in *Saccharomyces cerevisiae* have defined the *RAD52* epistasis group of genes which is believed to represent a pathway for the repair of DNA double-strand breaks (DSBs) by homologous recombination (Game and Mortimer, 1974; reviewed in Petes *et al.*, 1991; Game, 1993; Friedberg *et al.*, 1995; Shinohara and Ogawa, 1995). Among the *RAD52* group, the *rad51*, *rad52* and *rad54* mutants exhibit more severe repair and recombination defects, suggesting that these genes encode the central components of the recombinational repair mechanism. Inactivation of the *RAD51* gene renders *S.cerevisiae* as well as *S.pombe* cells highly sensitive to γ-rays, and strongly reduces both spontaneous and induced mitotic recombination frequencies (Saeki *et al.*, 1980; Shinohara *et al.*, 1992; Muris *et al.*, 1993).

Recently, structural homologues of the *RAD51*, *RAD52* and *RAD54* genes of *S.cerevisiae* have been cloned from vertebrates (Bezzubova *et al.*, 1993a,b; Morita *et al.*, 1993; Shinohara *et al.*, 1993; Bendixen *et al.*, 1994; Muris *et al.*, 1994; Kanaar *et al.*, 1996). The biochemical properties of human Rad51 are similar to those of *S.cerevisiae* Rad51 (Benson *et al.*, 1994; Sung and Robberson, 1995; Baumann *et al.*, 1996). Both the chicken B lymphocyte line, DT40 (Baba *et al.*, 1985) and mouse embryonic stem (ES) cells deficient in the *RAD54* gene are extremely sensitive to γ-ray irradiation, suggesting that the repair function of the RAD52 epistasis group genes is conserved throughout evolution (Bezzubova *et al.*, 1997; Essers *et al.*, 1997). However, the *RAD51* gene is essential for early murine embryonic development (Lim and Hasty, 1996; Tsuzuki *et al.*, 1996). Similarly, while DT40 clones deficient in the RAD54 gene can proliferate, RAD51 null mutants have not been successfully isolated from DT40 (Vainio and Imhof, 1995). Although these observations indicate that Rad51 is necessary for the survival of vertebrate cells, the essential role of the Rad51 protein in vertebrate cells has not yet been defined.

The following evidence suggests that Rad51 is expressed mainly in dividing cells. First, all dividing cell lines analyzed express RAD51 transcripts (Yamamoto *et al.*, 1996). Secondly, the amount of RAD51 transcripts is correlated with the extent of cell cycling among various tissues in chicken and mammals (Bezzubova *et al.*, 1993a; Shinohara *et al.*, 1993). Thirdly, Tashiro *et al.* (1996) showed that the expression of Rad51 protein is induced in human peripheral blood lymphocytes (PBLs) when the cells are stimulated with phytohemagglutinin (PHA). In addition, several nuclear foci containing Rad51 protein were observed in each S phase PBL (Tashiro *et al.*, 1996). The induction of similar foci by γ- and UV-irradiation (Haaf *et al.*, 1995) suggests that the function of Rad51 during cell cycling is related to that of Rad51 following irradiation.

Here, we describe the development of a genetic system in the chicken B lymphocyte line DT40 (Baba *et al.*, 1985; Buerstedde *et al.*, 1990) to study the essential function of Rad51. The high level of homologous recombination (Buerstedde and Takeda, 1991; Takeda *et al.*, 1992; Bezzubova and Buerstedde, 1994) in DT40 cells allowed us to perform targeted disruption of both RAD51 alleles. Furthermore, we created DT40 clones where human Rad51 protein (HsRad51) was expressed under the control of a repressible promoter (Wang *et al.*, 1996). We show that depletion of Rad51 is accompanied by an accumulation of cytologically detectable chromosome aberrations and subsequent cell death. Most of the detectable chromosome aberrations were isochromatid-type gaps and breaks, where both sister chromatids of a single chromosome are broken at the same locus, although we very occasionally detected chromatid-type gaps and breaks, where a single chromatid is broken. Since small numbers of random chromosome aberrations cannot be

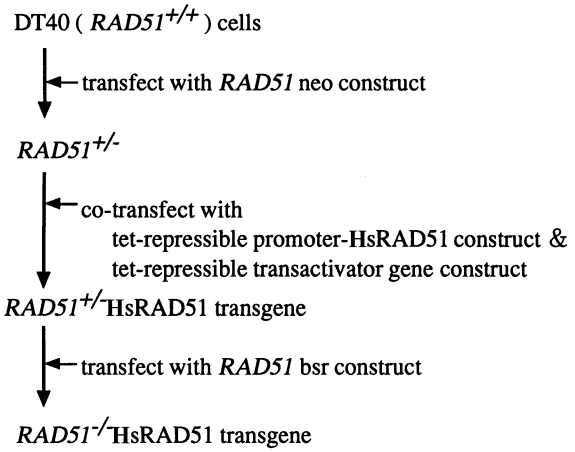

Fig. 1. Experimental strategy.

identified in yeast cells, our study defines for the first time the role of Rad51 in the maintenance of chromosomal DNA during normal cell cycling.

Results

Strategy of RAD51 disruption

Figure 1 shows the experimental procedure for generating conditional RAD51 mutant clones. In order to disrupt both alleles of the *RAD51* gene, we prepared two *RAD51* targeting constructs containing either the neomycin (neo) or blasticidin (bsr) resistance gene (see Figure 2A). First, we transfected the *RAD51*neo construct into DT40 cells and isolated clones heterozygous for the *RAD51* gene (*RAD51*$^{+/-}$). Secondly, one of the *RAD51*$^{+/-}$ clones was transfected with conditional human Rad51 expression constructs to obtain *RAD51*$^{+/-}$ cells carrying the constructs at random sites on the chromosome (*RAD51*$^{+/-}$/HsRAD51). Thirdly, we transfected the *RAD51 bsr* construct into several *RAD51*$^{+/-}$/HsRAD51 clones to isolate *RAD51*$^{-/-}$/HsRAD51 clones.

RAD51 targeting and expression constructs

A chicken *RAD51* (*GdRAD51*) cDNA probe (Bezzubova *et al.*, 1993a) was used to isolate genomic clones of the *RAD51* locus, which were partially sequenced to determine the positions of exons. About 5.5 kb of the GdRAD51 locus was then amplified by long-range PCR using genomic DNA from DT40 as a template. Either the neo or bsr resistance gene was inserted as shown into the genomic sequence (Figure 2A). Targeted integration of these constructs disrupts the reading frame of the Rad51 gene at amino acid position 80 (Bezzubova *et al.*, 1993a). Targeting events were recognized by Southern blot analysis of *Eco*RI-digested DNA using an external probe, by the appearance of 11 and 7.5 kb bands after targeted integration of the neo or bsr constructs, respectively (Figure 2A and B).

A conditional RAD51 expression construct was made by placing the human *RAD51* cDNA under the control of a tetracycline (tet)-repressible promoter (Gossen and Bujard, 1992). It has been previously demonstrated that the system allows tightly regulated gene expression in DT40 cells (Wang *et al.*, 1996).

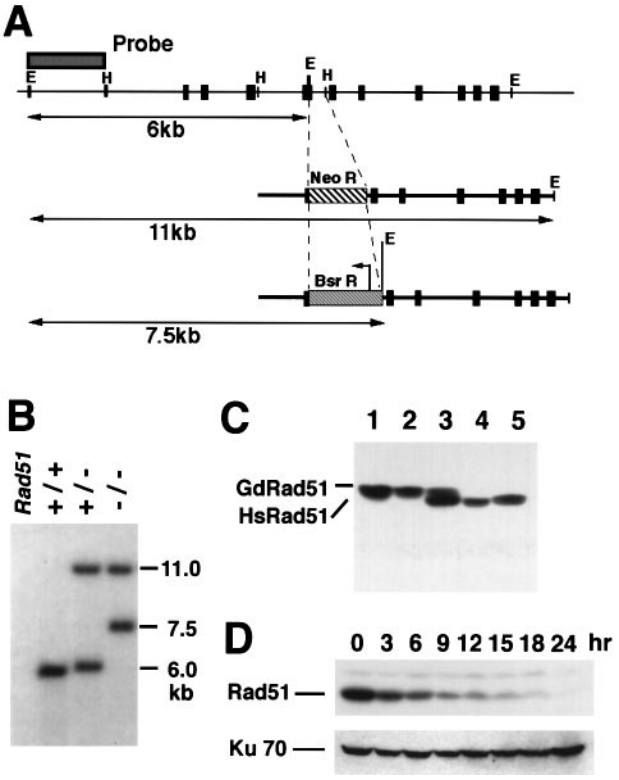

Fig. 2. Generation of a *RAD51*$^{-/-}$ clone carrying a human RAD51 transgene under the control of a tet repressible promoter.
(**A**) Schematic representation of part of the *RAD51* locus, the two gene disruption constructs and the configuration of the targeted loci. Closed boxes indicate the positions of exons deduced from the positions of primers in the cDNA sequence and from the sizes of PCR fragments. Relevant *Eco*RI and *Hin*dIII recognition sites and the position of the probe used in Southern blot analysis are indicated. New 11 kb and 7.5 kb *Eco*RI fragments are expected to hybridize with the probe following targeted integration of the indicated knockout constructs. (**B**) Southern blot analysis of *Eco*RI-digested DNA from wild-type DT40 (lane 1), a *RAD51*$^{+/-}$ (lane 2) and #110 *RAD51*$^{-/-}$ (lane 3) clone using the probe shown in (A). (**C**) Western blot analysis of the indicated cell extracts using anti-Rad51 antiserum. The following samples were loaded on each lane of a SDS–polyacrylamide gel: wild-type DT40 (lane 1), the *RAD51*$^{+/-}$ (lane 2), a *RAD51*$^{+/-}$ clone carrying the human Rad51 transgene (lane 3), #110 *RAD51*$^{-/-}$ clone (lane 4) and a human B lymphocyte line Ramos (lane 5). (**D**) Suppression of Rad51 expression from the transgene. Whole-cell lysates were prepared from *RAD51*$^{-/-}$ #110 cells at times indicated following the addition of tet. The filter was rehybridized with anti-Ku70 antibody to control for loading difference. Western blot analysis was performed in the same manner as in Figure 3C.

Generation of Rad51-deficient DT40 clones

The *RAD51 neo* construct was transfected into DT40 cells and drug-resistant clones were examined by Southern blot analysis to isolate heterozygous *RAD51*$^{+/-}$ mutant clones (Figure 2B). One of the *RAD51*$^{+/-}$ mutant clones was then transfected with the human Rad51 expression construct and with a second construct encoding the tet-responsive transcriptional activator as well as a hygromycin resistance marker. To test for tet-repressible synthesis of human Rad51, hygromycin-resistant clones were grown for 48 h in the presence and absence of tet, and whole-cell lysates were prepared and screened by Western blotting using anti-human Rad51 antiserum (Tashiro *et al.*, 1996). Chicken Rad51 is 95% identical to human Rad51 at the amino acid level (Bezzubova *et al.*, 1993a), cross-reacts with the anti-human Rad51 antiserum, and migrates

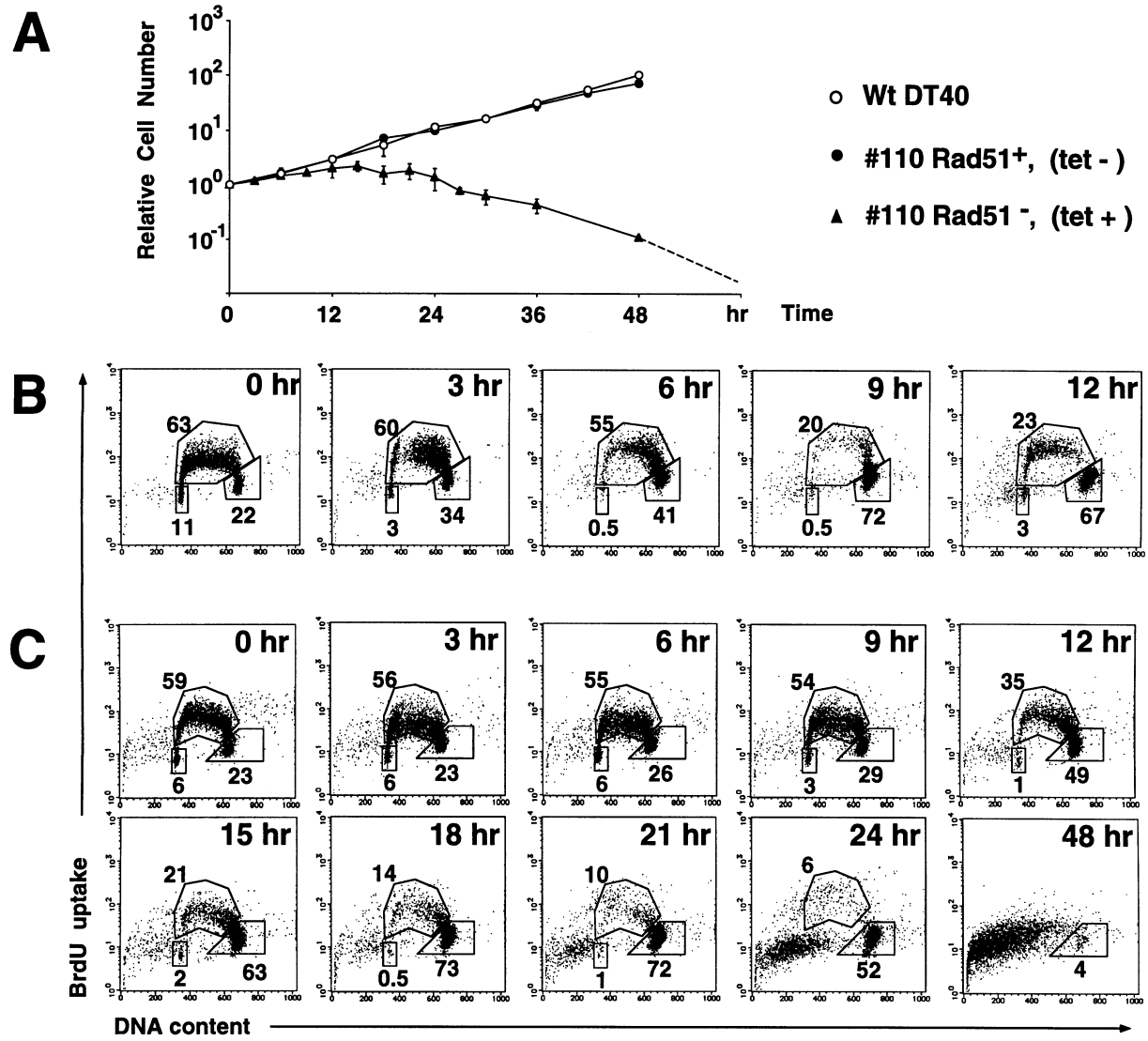

Fig. 3. Cell cycle analysis of wild-type cells and the #110 *RAD51*$^{-/-}$ clone. (**A**) Representative growth curves corresponding to the indicated cell cultures. Tet was added at time zero to the #110 tet (+) culture. The numbers of cells not stained with trypan blue were counted. Each experiment was performed three times, and each time point was determined in triplicate. (**B**) Cell-cycle distribution of wild-type DT40 cells at indicated times following irradiation with a dose of 4-Gy γ-ray. Cells were stained with FITC-anti-BrdU (*y*-axis, log scale) to detect BrdU incorporation and with propidium iodide to detect total DNA (*x*-axis, linear scale). The lower-left box identifies G_1 cells, the upper box identifies S cells, and the lower-right box identifies G_2/M cells. A single dose of irradiation was given at time zero. The numbers given on the boxes indicate the percentages of gated events. (**C**) Cell cycle analysis of #110 *RAD51*$^{-/-}$ cells following the inhibition of HsRAD51 transgene expression by adding tet at time zero.

slightly more slowly than human Rad51 in an SDS gel (Figure 2C). *RAD51*$^{+/-}$ mutant clones displaying efficient tet-repressible expression of the HsRAD51 transgene were transfected with the *RAD51 bsr* construct (Figure 2A) to disrupt the second *RAD51* allele. Several homozygous *RAD51*$^{-/-}$ mutant clones were obtained and one, named #110, was chosen for further analysis. The amounts of human Rad51 protein per cell were comparable between #110 and a representative human cell line: an Epstein–Barr virus-transformed B lymphocyte line called Ramos (Myers *et al.*, 1995). Western blotting using the anti-human Rad51 antibody (Figure 2C) showed that only human Rad51, but not chicken Rad51, was expressed in the #110 clones.

Western blot showed that #110 clones displayed steady reduction of human Rad51 with time in the presence of tet (Figure 2D). The half-life of human Rad51 was ~3 h. We next examined cell growth and viability following the addition of tet to the medium.

Deletion of Rad51 results in an accumulation of cells in the G_2/M phase and subsequent cell death

The proliferative properties of HsRad51$^+$ #110 cells (tet$^-$) were monitored by growth curves and by cell cycle analysis. The growth curve of #110 was indistinguishable from wild-type DT40, which divided every ~8 h (Figure 3A). The cell cycle was analyzed by pulse-BrdU-labeling followed by cytofluorometric analysis. Asynchronous wild-type DT40 and #110 showed similar patterns (compare 0 h in Figure 3B and C) with the S phase accounting for two-thirds of the whole cell cycle time.

In order to analyze the effect of DSB formation on the genome, wild-type DT40 cells were irradiated with 4-Gy γ-ray, which inhibits the subsequent exponential prolifer-

Table I. Mitotic indices of wild-type and Rad51 deficient DT40 cells in the presence or absence of tet

Cells	Treatment	Colcemid (–) (%)	Colcemid (+) (%)
wild-type	–	2.4	21
#110	–	3.9	19
#110	tet	3.2	33

Wild-type and Rad51-deficient DT40 cells were cultured with (+) or without (–) colcemid for 3 h. #110 Rad51 were cultured with or without tet for 21 h. The values represent the average percentages of mitotic cells in two experiments.

ation of 70–80% of the irradiated cells (Bezzubova *et al.*, 1997). Upon irradiation, cells accumulated in the G_2/M phase but not in the G_1 phase (Figure 3B). #110 cells exhibited essentially the same pattern of response to γ-irradiation as wild-type cells (data not shown). These observations suggest that in DT40, the G_1 checkpoint does not function while the G_2 checkpoint works normally. Lack of G_1 arrest may be caused by the absence of p53 expression in DT40 (data not shown), as consistently observed in other chicken cell lines (Ulrich *et al.*, 1992). Cells either died or began cycling normally at 12 h post-irradiation. The induction of apoptosis in DT40 appears to be normal, because the cross-linking of surface immunoglobulin receptors (Takata *et al.*, 1995), the removal of fetal calf serum from the media and γ-irradiation (Uckun *et al.*, 1996) induce the rapid apoptosis of DT40 cells, as observed in other mammalian lymphocyte lines (Cohen *et al.*, 1992).

The proliferative properties of Rad51-deficient #110 cells (tet^+) were monitored by growth curves and by cell cycle analysis. The cells ceased proliferating at ~12 h following the addition of tet (Figure 3A), and no cells survived at 72 h. Tet had no effect on the growth or viability of wild-type cells (data not shown) as previously described (Wang *et al.*, 1996). These observations indicated that the depletion of Rad51 resulted in growth arrest and subsequent cell death. Cell cycle analyses revealed that cells began to accumulate in the G_2/M phases at 12 h after the addition of tet (Figure 3C). Subsequently, the degradation of chromosomal DNA was observed due to massive cell death between 24 and 48 h (Figure 3C).

Cell cycle analysis showed that 23% of the $HsRad51^+$ #110 cells (tet^-) were in the G_2/M phases (Figure 3C, 0 h) while the mitotic index of the cells was 3.9% (Table I). On the other hand, at 21 h after the addition of tet, the percentage of #110 cells in the G_2/M phase was elevated to 72% while the mitotic index in this culture was only 3.2%. These observations indicate that Rad51-deficient #110 cells were arrested at the G_2/M boundary, as observed in irradiated cells (Figure 3B).

When Rad51-deficient #110 cells were treated with colcemid from 18 until 21 h after the addition of tet, the percentage of metaphase-blocked mitotic cells was elevated to 33% from 3.2% (Table I). Colcemid treatment of wild-type cells as well as $HsRad51^+$ #110 cells elevated the percentages of mitotic cells to a similar extent. These results suggest that a substantial fraction of Rad51-deficient #110 cells had passed through the G_2/M checkpoint and were capable of initiating mitosis with kinetics similar to those of wild-type and $HsRad51^+$ #110 cells.

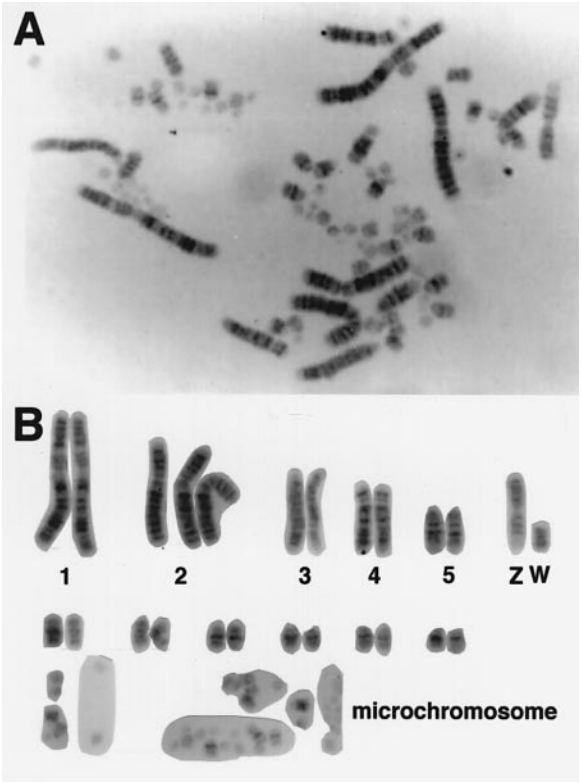

Fig. 4. Karyotype analysis of DT40 cells. Chromosomes spread on a glass slide were photographed (**A**) and subsequently aligned as shown in (**B**). Macrochromosomes 1–5 and Z are identified.

Rad51-deficient cells display highly elevated frequencies of chromosomal breakage

DT40 cells display a stable karyotype with a modal chromosome number of 80, which comprises 11 autosomal macrochromosomes, the ZW sex chromosomes and 67 microchromosomes (Figure 4). The karyotype does not show obvious abnormalities except for a trisomy of chromosome 2 and one additional microchromosome, which were consistently observed in DT40 cells (Figure 4). Since changes in the microchromosomes are difficult to judge, subsequent analysis of chromosomal structural aberrations was limited to the 11 autosomal macrochromosomes and the Z chromosome in conventionally Giemsa-stained metaphase cells. The numbers of these chromosomes per cell were highly stable in wild-type and #110 $Rad51^+$ cells (Figure 5). In addition, chromosome loss was not observed in #110 $Rad51^-$ cells in the presence of tet (Figure 5). The scoring criteria were essentially the same as those of ISCN (ISCN1985, 1985). According to ISCN, a break is defined as a discontinuity of a chromosome that shows a clear misalignment of the distal fragment of a broken chromosome. A gap is defined as a clear non-staining region on a chromosome.

Chromosomal aberrations were hardly detectable in wild-type as well as $Rad51^+$ #110 cells (Table II, first and fourth row). Also, the addition of tet to wild-type cells did not affect the frequency of aberrations (data not shown). Three hours after 4-Gy γ-irradiation of asynchronous wild-type cells, chromatid-type gaps (small arrowheads in Figure 6B) and breaks (large arrowheads in Figure 6B) were frequently observed (Table II, second row). They may reflect the generation of DSBs after DNA

replication. At 12 h post-irradiation, detectable structural aberrations were mostly isochromatid-type gaps (small arrows in Figure 6C) and breaks (large arrows in Figure 6C), indicating that the cells had been in the G_1 or S phase at the time of γ-irradiation (Table II, third row). These observations showed that, as previously reported (Evans, 1962), the types of chromosomal aberrations reflect the phases of cell cycling during which DSBs were generated by γ-irradiation. Chromatid exchange, which is the result of two or more chromatid lesions and the subsequent rearrangement at chromatid level (ISCN1985, 1985), was also frequently detectable at 12 h post-irradiation (Table II).

In the following studies, we analyzed metaphase-arrested #110 cells that had been incubated in medium containing colcemid for 3 h. The karyotype of #110 cells is quite stable, as is that of wild-type DT40 cells. In HsRad51$^+$ #110 cells, only three chromosomal aberrations were detectable among 200 mitotic cells and were all chromatid breaks (Table II, fourth row). Chromosomal aberrations were first detectable at 9 h following the addition of tet (Table II, sixth row). In marked contrast to wild-type cells at 3 h after γ-irradiation (Table II, second row), Rad51-deficient #110 cells showed isochromatid-type gaps and breaks (Figure 6 E) but only rarely chromatid-type aberrations at 12 h. Similarly, at 15, 18 and 21 h following the addition of tet, #110 cells showed isochromatid-type breaks much more frequently than chromatid-type aberrations.

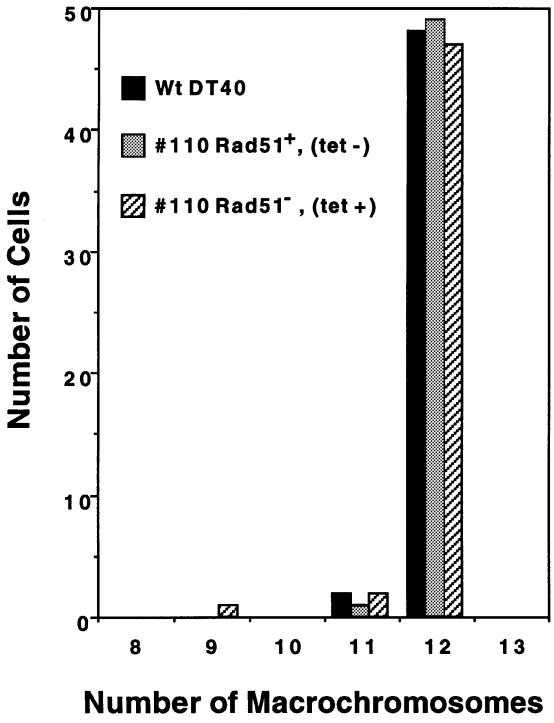

Fig. 5. Distribution of the number of macrochromosomes per cell. Wild-type DT40 cells were cultured with colcemid for 3 h. Rad51-deficient DT40 cells were cultured with or without tet for 21 h, and with colcemid for the last 3 h. The number of macrochromosomes (1–5 and Z) were scored in 50 metaphase cells.

At 21 h following the addition of tet, a total of 222 chromosomal aberrations were found in 200 #110 cells, i.e. 1.1 aberrations per cell (Table II). It should be noted that we may have underestimated the number of chromosomal aberrations because of our limited scoring criteria for aberrations. Since we scored chromosomal aberrations observed only on macrochromosomes, which account for ~75% of the chicken genomic DNA (Bloom *et al.*, 1993), the average number of aberrations per cell was calculated to be on average ~1.5 per cell (1.1/0.75).

The number of aberrations per cell showed a Poisson distribution (Table IIIA), suggesting that chromosomal aberrations had occurred randomly in the population of analyzed Rad51$^-$ cells. In addition, chromosomal aberrations appeared to distribute randomly on the chromosomes because the relative numbers of aberrations on each chromosome were nearly proportional to the relative length of each macrochromosome (Table IIIB). These observations indicated that chromosomal aberrations of Rad51$^-$ cells occurred in a stochastic manner.

Discussion

The recombinational repair pathway may be required to repair DSBs in proliferating vertebrate cells

We report here the phenotype caused by the disruption of the RAD51 gene in the chicken B-cell line DT40. Rad51-deficient cells became arrested in the G_2/M phase of the cell

Table II. Frequencies of chromosomal aberrations in wild-type and Rad51 deficient DT40 cells

Cells	Treatment	Time after treatment (h)	No. of cells analyzed	Chromatid		Isochromatid		Chromatid exchanges	Total (per cell)
				Gaps	Breaks	Gaps	Breaks		
Wild-type	–		100	4	0	0	0	0	4 (0.04)
	4 Gy	3	100	2	9	5	5	2	23 (0.23)
	4 Gy	12	100	0	5	5	33	8	51 (0.51)
#110	–		200	3	0	0	0	0	3 (0.02)
	tet	6	200	2	0	1	0	0	3 (0.02)
	tet	9	200	4	0	0	4	0	8 (0.04)
	tet	12	200	3	0	9	56	1	69 (0.35)
	tet	15	200	1	0	7	84	0	92 (0.46)
	tet	18	200	4	0	2	111	0	117 (0.59)
	tet	21	200	3	0	9	207	3	222 (1.11)

Wild-type DT40 cells were irradiated with 4 Gy γ-ray and chromosome aberrations were scored in 100 metaphase cells at 3 and 12 h post-irradiation. The #110 Rad51$^+$ cells were cultured with tet, and chromosome aberrations were scored in 200 metaphase cells at time indicated after addition of tet. All samples were treated with colcemid for the last 3 h.

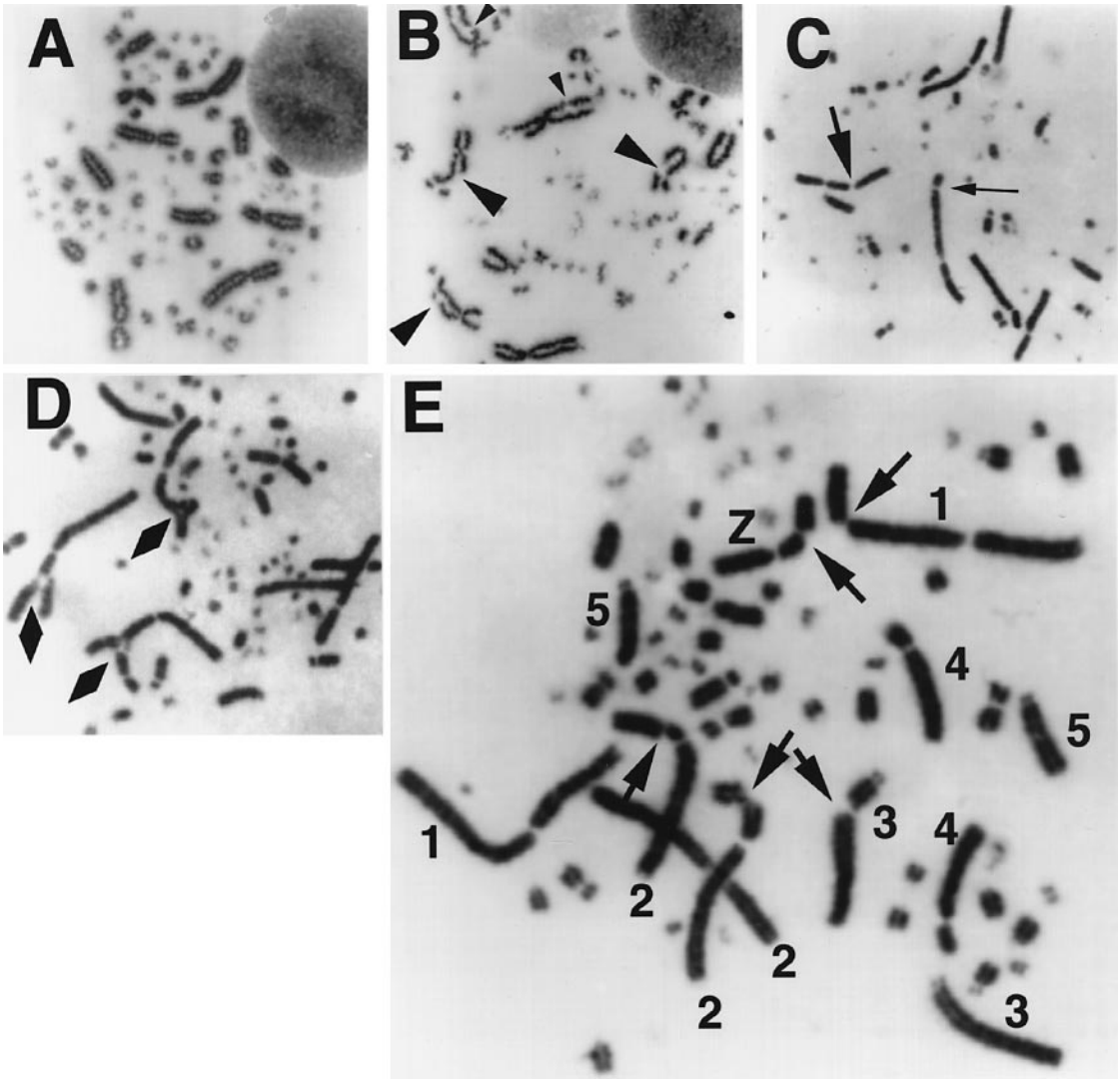

Fig. 6. Representative karyotype analysis of 4-Gy-irradiated wild-type cells and Rad51-deficient #110 cells. (**A**) Representative karyotype of wild-type DT40 cells. (B–D) Chromosome aberrations in wild-type cells following 4-Gy irradiation. Karyotype was analyzed at 3 h (**B**) and 8 h (**C** and **D**) following irradiation. (**E**) Karyotype of Rad51^{-} #110 cells after incubation in the tet^{+} medium for 21 h. Macrochromosomes 1–5 and Z are identified. Chromatid breaks and gaps are shown by small and large arrowheads, respectively. Isochromatid gaps and breaks are shown by small and large arrows, respectively. Exchanges are shown by rhombi.

cycle, accumulating cytologically visible chromosomal breaks, and eventually dying. Given the known role of yeast Rad51 in recombinational repair of DSBs of the genome (Shinohara *et al.*, 1992; Shinohara and Ogawa, 1995), chromosomal damage may also be repaired through the recombinational repair pathway involving Rad51 in normal vertebrate cells.

Genetic studies of yeast mutants revealed that Rad51 is essential to process induced DSBs prior to homologous recombination in meiosis as well as the mating type switch (Shinohara *et al.*, 1992; Haber, 1997). Also, Rad51 has been shown to play an essential role in repairing DSBs generated by X-irradiation (reviewed in Petes *et al.*, 1991; Game, 1993; Friedberg *et al.*, 1995; Shinohara and Ogawa, 1995). Given the role of Rad51 in DSB repair in yeast, the cytogenetically visible chromosome breaks may reflect deficient repair of DSBs.

RAD52 epistasis group mutants from *S.cerevisiae* as well as from *S.pombe* display an increased rate of chromosome loss (Morrison and Hastings, 1979; Mortimer *et al.*, 1981; Muris *et al.*, 1996), and multiple chromosome loss was reported in early mouse embryos deficient in Rad51 protein (Lim and Hasty, 1996). Evidence for chromosome loss was not obtained in Rad51-deficient DT40 cells (Figure 5). These previous observations are, however, not inconsistent with our data, because chromosomal breaks can be converted into chromosome loss in daughter cells after multiple rounds of mitosis (Grote *et al.*, 1981a,b).

Chromosomal aberrations may cause cell death in Rad51-deficient cells

The presence of a single unrepaired DSB is sufficient to induce cell death in yeast, as shown in the inducible expression of the HO nuclease in radiation-sensitive yeast mutants (Game, 1993). Similarly, rapidly accumulating chromosomal breaks may cause the rapid death of Rad51-deficient DT40 cells. However, although Rad51-deficient cells were not viable, substantial numbers of the cells did not manifest structural aberrations of the chromosomes. This observation suggested that not all lethal DNA lesions

Table III. Distribution of chromosomal aberrations in #110 Rad51-deficient DT 40 cells at 21 h following addition of tet

(A) Frequency of chromosome aberrations

No. of aberrations	0	1	2	3	4	5	6	<7	Total
No. of cells with indicated No. of aberrations									
Observed	58	83	43	12	1	2	1	0	200
Expected[a]	64	72	40	16	8	2	–	–	202

Chromosomal aberrations were scored in 200 metaphase cells.
[a]Expected from Poisson distribution. The distribution shows a good fit to Poisson distribution ($\chi^2 = 8.32$, d.f. = 4, $P = 0.08$).

(B) Distribution of chromosome aberrations among macrochromosomes

Chromosomes	1	2	3	4	5	Z	Total
No. of chromatid gap/break	0/0	1/0	0/0	1/0	0/0	0/0	2/0
No. of isochromatid gap/break	4/74	1/67	0/29	1/25	0/5	1/11	6/211
No. of chromatid exchange	0	2	0	0	0	1	3
Total No. of aberrations (observed)	78	71	29	26	5	13	222
Total No. of aberrations (expected)[a]	61	67	33	28	18	14	221

Chromosomal aberrations were scored in 200 metaphase cells.
The distribution of chromosome aberrations among chromosomes is nearly proportional to the relative length of chromosome ($\chi^2 = 15.0$, d.f. = 5, $P = 0.011$). Deviation from the randomness is mainly due to an excess involvement of chromosome 1 and less involvement of chromosome 5 compared with the expected values.
[a]Expected from the relative length of chromosomes.

were necessarily manifested as chromosomal aberrations. Moreover, we could only analyze the chromosomes of the cells that had passed through the G_2/M checkpoint, and, as a result, we probably underestimate the number of chromosomal aberrations. The number of Rad51 foci observed in the S phase of vertebrate cells, i.e. five per cell on average (Tashiro *et al.*, 1996), may reflect the actual number of DNA lesions in Rad51-deficient cells.

The present data suggest that unrepaired lesions on the genome were responsible for the subsequent cell death of Rad51-deficient cells. Nonetheless, we cannot exclude other mechanisms for the death of Rad51-deficient cells. It is possible that through other, unknown mechanisms, lack of Rad51 protein caused apoptosis that in turn initiated DSBs. This possibility is, however, not likely for the following reasons. First, the observed elevation of mitotic index following colcemid treatment (Table I) suggests that cells carrying chromosomal breaks were capable of entering mitosis. Next, apoptosis is expected to trigger rapid biochemical chain reactions to activate non-specific digestion of chromosomal DNA (Fraser and Evan, 1996; Nagata, 1997). Such rapid reactions would digest chromosomal DNA in metaphase-arrested cells and would not result in the Poisson distribution of chromosomal aberrations (Table IIIA). Furthermore, the presence of isochromatid-type breaks is inconsistent with apoptosis, which would trigger random digestion of the genome.

Previous reports have shown that Rad51 is essential for the survival of ES cells and for early embryogenesis (Lim and Hasty, 1996; Tsuzuki *et al.*, 1996). In marked contrast to these findings, all of the *RAD52* group mutants from yeast species are viable, though Rad51 mutants grow slowly with poor plating efficiency (Morrison and Hastings, 1979; Mortimer *et al.*, 1981; Fingerhut *et al.*, 1984; Muris *et al.*, 1993; Muris *et al.*, 1996). Given that vertebrate cells carry genomes hundreds of times larger than yeast cells, spontaneous DSBs should occur much more frequently in a vertebrate cell than in a yeast cell. Hence, the difference in genome might explain how the absence of Rad51 causes cell death in vertebrate but not in yeast cells.

Chromosomal breaks may occur during DNA replication in Rad51-deficient cells

It is known that DSBs occurring prior to and during DNA replication result in isochromatid breaks while DSBs generated following DNA replication result in chromatid breaks. Accordingly, after γ-irradiation of asynchronous cells, chromatid-type breaks appeared earlier than isochromatid-type breaks in mitotic cells (Table II). In contrast, isochromatid-type breaks were consistently observed in Rad51-deficient mitotic cells from 12 to 21 h following the addition of tet (Table II). This finding suggests that recombinational repair and chromosomal breaks are associated with DNA replication. This notion is supported by the S phase-specific appearance of Rad51 foci (Tashiro *et al.*, 1996). On the other hand, it is not likely that Rad51 functions prior to DNA replication since it is involved in recombinational repair, in which damaged chromosomal DNA interacts with the other intact sister chromatid or heteroallelic chromosome through homologous DNA recombination. Homologous recombination between heteroallelic chromosomes should be extremely rare in vertebrate cells in order to maintain the heterozygosity of the genome.

The following studies of yeast mutants indicate a role of the *RAD52* epistasis group genes in DNA replication. First, mutations in the *RAD27* gene are lethal in combination with mutations in either the *RAD51* or *RAD52* gene. The *RAD27* of *S.cerevisiae* and *RAD2* of *S.pombe* are structural homologues of mammalian *FEN-1*, which is required for *in vitro* DNA replication (Ishimi *et al.*, 1988; Kenny *et al.*, 1988; Goulian *et al.*, 1990; Turchi and Bambara, 1993; Harrington and Lieber, 1994; Waga *et al.*, 1994). This suggests that the majority of replication errors that accumulate in *RAD27*-deficient strains are repaired

by the recombinational repair pathway (Murray *et al.*, 1994; Tishkoff *et al.*, 1997). Secondly, the elevated rate of deletion of genomic sequences accompanying a mutation in DNA polymerase δ can be suppressed by another mutation in either RAD50 or RAD52 (Gordenin *et al.*, 1992; Tran *et al.*, 1995). Thirdly, the viability of *S.pombe* strains carrying mutations in *RAD51* or *RAD54* homologues is drastically reduced when mutations affecting S phase checkpoint are added (Muris *et al.*, 1996). The presence of isochromatid breaks in Rad51-deficient DT40 cells, therefore, supports the notion that recombinational repair is a normal consequence of DNA replication in vertebrate cells. While 'RAD' genes have been known to repair DNA lesions induced by irradiation, our data indicate that Rad51 plays an essential role in repairing DNA lesions generated during normal DNA replication in vertebrate cells.

Possible mechanism for the formation of isochromatid breaks during DNA replication

It was recently reported that the Holliday junction recombination intermediates accumulated spontaneously during DNA replication in mitotically growing yeast and that specific replication defects led to an increase in Holliday junctions (Zou and Rothstein, 1997). The formation of Holliday junctions was shown to be dependent upon the function of a recombination protein, Rad52, but surprisingly independent of Rad51. This observation suggests that DNA lesions generated during DNA replication stimulate the recombinational repair pathway and that recombination intermediates can be formed after DNA replication between damaged and the other intact sister chromatids even in the absence of Rad51. Recombinational repair can be stimulated by at least three types of DNA lesions encountered by the replication machinery (Zou and Rothstein, 1997): a nick (Skalka, 1974), bulge [for review, see (Kogoma, 1997)] and mismatch (Borts *et al.*, 1990; Zgaga *et al.*, 1991). When a replication fork encounters a nick on the template, the fork is interrupted, forming a pair of intact and broken chromatids but not isochromatid-type breaks (Skalka, 1974). As a result, the absence of recombinational repair would lead to an increase in the number of chromatid breaks but not isochromatid breaks. It is, therefore, surprising that Rad51-deficient DT40 cells exhibited isochromatid-type breaks without increasing the number of chromatid breaks. Hence, we postulate that the absence of Rad51 in DT40 may cause observed isochromatid-type breaks in the following manner. In Rad51-deficient DT40, the presence of DNA lesions following DNA replication is capable of stimulating recombinational repair, forming Holliday junctions as observed in yeast cells (Zou and Rothstein, 1997). Some Holliday junctions may not be processed normally during resolution causing isochromatid breaks. In contrast, since DT40 clones deficient in either Rad52 (Y.Yamaguchi-Iwai, J.-M.Buerstedde, O.Bezzubova, A.Shinohara, H.Ogawa, M.Takata, E.Sonoda and S.Takeda, unpublished data) or Rad54 (Bezzubova, 1997) are capable of proliferating, the absence of these proteins may inhibit the formation of the Holliday junctions or may not disturb the resolution of the Holliday junctions.

Biological significance of the spontaneous occurrence of chromosomal breaks in cycling vertebrate cells

Sister chromatid exchanges (SCEs) are observed in normally cycling vertebrate cells (Perry and Wolff, 1974), *Drosophila* (Tsuji and Tobari, 1979) as well as plant cells (Schvartzman *et al.*, 1978). The frequencies of SCEs are elevated following various genotoxic treatments. It has been speculated that SCEs occur as the result of DNA strand exchange during S phase. The present data imply that the generation of strand breaks during DNA replication is followed by recombinational repair of the lesion, which eventually results in a gene conversion or exchange with the other intact sister chromatid.

Such spontaneous formation of DSBs in vertebrate cells suggests a mechanism for chromosomal translocation. In addition, repair of such DSBs may mediate targeted integration of transfected DNA through homologous DNA recombination since the introduction of DSBs into the genome elevates the frequencies of targeted integration in mammalian cells (Rouet *et al.*, 1994).

Nuclear injection of linearized plasmid DNA revealed that the presence of a single DSB may be sufficient to induce a p53-dependent cell cycle arrest (Huang *et al.*, 1996). Assuming extremely sensitive recognition of DSBs, it should be investigated how cells can distinguish DNA lesions generated during normal cell cycling from DNA lesions caused by genotoxic treatment.

In recent studies, Rad51 protein from mammalian cells was reported to interact with the tumor suppressor protein p53 (Sturzbecher *et al.*, 1996) and to be associated with the RNA polymerase II transcription complex (Maldonado *et al.*, 1996), Brca1 (Scully *et al.*, 1997) and Brca2 (Mizuta *et al.*, 1997; Sharan *et al.*, 1997). The specificity and functional nature of these interactions remains unknown, but may point to a pleiotropic role for Rad51 in DNA metabolism.

Materials and methods

Plasmid constructs

The *RAD51 neo* disruption construct was made by cloning the neo^R gene in-frame into the *RAD51* coding sequence, so that the neo^R gene was expressed under the control of the endogenous *RAD51* gene promoter. The left and right arms of the disruption constructs were derived from 1.1 and 4.2 kb *Hin*dIII–*Eco*RI fragments of chromosomal DNA, respectively, as shown in Figure 2. For the *RAD51 bsr* disruption construct, a blasticidin resistance (Bsr^R) gene containing a promoter derived from Molony murine leukemia virus (MoMuLV) LTR was inserted between the two arms. This Bsr^R gene consisted of a *Cla*I–*Eco*RI fragment of the 5′LTR from a retrovirus vector (Eglitis *et al.*, 1985) and the *Eco*RI–*Bam*HI fragment of Bsr^R gene (Takami *et al.*, 1997) followed by a polyadenylation signal in the *Hin*dIII–*Eco*RI fragment of pAGS-3 (Miyazaki *et al.*, 1989). The *RAD51 Neo* and Bsr disruption constructs were linearized with *Eco*RI and *Not*I, respectively, prior to electroporation. Targeted integration of these constructs was expected to disrupt the RAD51 gene after amino acid position 80 (Bezzubova *et al.*, 1993a). To construct a plasmid for the inducible expression of HsRad51, the human *RAD51* cDNA (Shinohara *et al.*, 1992) was inserted between the *Sac*II (Klenow-treated) and *Bam*HI sites of a pUHG 10-3 vector (gift from H.Bujard, Heidelberg), in which a rabbit β-globulin intron and polyadenylation signal was inserted behind the polycloning sites of pUHG 10-3 (Gossen and Bujard, 1992). To construct a vector encoding both the tetracycline transactivator (tTA) and Hygromycin resistance (Hyg^R) genes; ptTA-Hyg^R (gift from K.Shimizu, Okayama), the *Xho*I–*Hin*dIII fragment containing the tTA gene from pUHD 15-1 (Gossen and Bujard, 1992) was ligated into the *Sal*I–*Hin*dIII

sites of a plasmid containing the Hyg^R gene under the control of the herpes simplex virus thymidine kinase gene (HSV tk) promoter (Mcknight, 1980). The pUHG 10-3 vector carrying the human *RAD51* transgene and the ptTA-Hyg^R construct were linearized with *Pvu*I and *Hin*dIII, respectively, prior to co-electroporation.

Cell culture, DNA transfections and irradiation

Cells were cultured in RPMI#1640 medium supplemented with 10^{-5} M β-mercaptoethanol, penicillin, streptomycin, 10% fetal calf serum and 1% chicken serum (Sigma, St. Louis, MO, USA) at 39.5°C. DNA transfection was performed as previously described (Buerstedde and Takeda, 1991). Briefly, 10^7 cells were suspended in 0.5 ml PBS containing 10–30 μg of linearized plasmid for each transfection and electroporated with a Gene Pulser apparatus (BioRad, Hercules, CA) at 550 V and 25 μF. Following electroporation, cells were transferred into 20 ml fresh medium and incubated for 24 h. Cells were then resuspended in 80 ml medium containing the appropriate drugs and divided into four 96-well plates. After 7–10 days, drug-resistant colonies were transferred to 24-well plates. Drug-resistant colonies were selected in 96-well microtiter plates with medium containing either 2 mg/ml geneticin (GIBCO-BRL, Grand Island, NY), 50 μg/ml blasticidin-S (Calbiochem, La Jolla, CA) or 2.5 mg/ml hygromycin (Calbiochem). Doxycycline (tet, Sigma) was used at the concentration of 10 ng/ml (Gossen *et al.*, 1995). γ-irradiation was performed using ^{137}Cs (0.02 Gy/s, Gammacell 40, Atomic Energy of Canada Limited Industrial Products, Ontario).

Western blot analysis

Cells (10^6) were washed with PBS and lysed in 20 μl SDS lysis buffer [25 mM Tris–HCl (pH 6.5), 1% SDS, 0.24 M β-mercaptoethanol, 0.1% Bromophenol Blue, 5% glycerol]. Following sonication and boiling, aliquots (routinely 50%) were subjected to 10% SDS–PAGE. After transfer to nylon membrane, proteins were detected by polyclonal rabbit anti-human Rad51 polyclonal serum (Tashiro *et al.*, 1996), rabbit anti-chicken Ku70 polyclonal serum (M.Takata, M.S.Sasaki, E.Sonoda, C.Morrison, Y.Yamaguchi-Iwai, A.Shinohara and S.Takeda, manuscript in preparation) and HRP-conjugated goat anti-rabbit Ig (Santa Cruz Biotechnology, CA) using Super Signal™ CL-HRP Substrate System (Pierce, Rockford, IL).

Cell cycle analysis

Cells were labeled for 10 min with 20 μM bromodeoxyuridine (BrdU; Amersham, Buckinghamshire, UK). They were then harvested and fixed at 4°C overnight with 70% ethanol, and incubated in the following ways: (i) in 4 N HCl, 0.5% Triton X-100 for 30 min at room temperature; (ii) in FITC-conjugated anti-BrdU antibody (Pharmingen, San Diego, CA) for 1 h at room temperature; (iii) in 5 μg/ml propidium iodide in PBS. Between each incubation, cells were washed with PBS containing 2% FCS and 0.1% sodium azide. Subsequent flow-cytometric analysis was performed on a FACScan (Becton Dickinson, Mountain View, CA). Fluorescence data were displayed as dot plots using the Cell Quest software (Becton Dickinson).

Chromosome aberration analysis

For trypsin G-banding (Figure 4), cells were treated for 1 h with medium containing 0.1 μg/ml colcemid (GIBCO-BRL, Grand Island, NY). Preparation of chromosome samples was done as previously described (Dracopoli, 1994). Briefly, harvested cells were treated in 1 ml of 0.9% sodium citrate for 15 min at room temperature and fixed in 5 ml of freshly prepared 3:1 mixture of methanol/acetic acid. The cell suspension was dropped onto an ice-cold wet slide glass and air dried. The slides were treated with 0.25% trypsin for 10 s, rinsed in PBS and then stained with 3% Giemsa solution at pH 6.4 for 10 min. To detect chromosomal aberrations (Figure 6), we modified this procedure as follows. Cells were treated for 3 h with medium containing 1 μg/ml colcemid. Since the chromatin condenses strongly with such a high concentration of colcemid, chromosomal gaps and breaks were more clearly detectable. Cell suspension was dropped onto an ice-cold wet glass slide and immediately flame dried. The trypsin treatment of slides was omitted.

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