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The *XRCC2* and *XRCC3* repair genes are required for chromosome stability in mammalian cells

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Abstract

The irs1 and irs1SF hamster cell lines are mutated for the *XRCC2* and *XRCC3* genes, respectively. Both show heightened sensitivity to ionizing radiation and particularly to the DNA cross-linking chemical mitomycin C (MMC). Frequencies of spontaneous chromosomal aberration have previously been reported to be higher in these two cell lines than in parental, wild-type cell lines. Microcell-mediated chromosome transfer was used to introduce complementing or non-complementing human chromosomes into each cell line. irs1 cells received human chromosome 7 (which contains the human *XRCC2* gene) or, as a control, human chromosome 4. irs1SF cells received human chromosome 14 (which contains the *XRCC3* gene) or human chromosome 7. For each set of hybrid cell lines, clones carrying the complementing human chromosome recovered MMC resistance to near-wild-type levels, while control clones carrying noncomplementing chromosomes remained sensitive to MMC. Fluorescence in situ hybridization with a human-specific probe revealed that the human chromosome in complemented clones remained intact in almost all cells even after extended passage. However, the human chromosome in noncomplemented clones frequently underwent chromosome rearrangements including breaks, deletions, and translocations. Chromosome aberrations accumulated slowly in the noncomplemented clones over subsequent passages, with some particular deletions and unbalanced translocations persistently transmitted throughout individual subclones. Our results indicate that the *XRCC2* and *XRCC3* genes, which are now considered members of the *RAD51* gene family, play essential roles in maintaining chromosome stability during cell division. This may reflect roles in DNA repair, possibly via homologous recombination.

Keywords: *XRCC2*; *XRCC3*; Chromosomal stability; Chromosome rearrangement; Human–hamster hybrid cells; DNA repair; Homologous recombination; Microcell-mediated chromosome transfer

1. Introduction

The X-ray repair cross complementing (XRCC) genes correct the phenotypes of certain mutated rodent cell lines for sensitivity to ionizing radiation and other DNA damaging agents. The human *XRCC2*

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and *XRCC3* genes were originally identified by their ability to complement the irs1 and irs1SF mutant cell lines isolated from V79 and AA8 Chinese hamster cells respectively [1–5]. The irs1 and irs1SF cell lines have similar phenotypes of sensitivity to DNA-damaging agents. Both are moderately sensitive to X-ray or gamma radiation (~ 2-fold), to UV radiation (2- to 3-fold), and to ethylmethanesulfonate (EMS, 2- to 10-fold) [1,2]. However, both show extreme sensitivity to DNA cross-linking agents, such as cisplatin and nitrogen mustard [6] and particularly mitomycin C (MMC, 60- to 100-fold) [1,3,4]. In addition, both irs1 and irs1SF suffer increased rates of spontaneous and X-ray-induced chromatid and chromosome breaks [2,3,7].

The complementing human genes have been cloned recently [3,8,9]. Sequence analysis of the human *XRCC2* and *XRCC3* genes has revealed that both bear homology to the *RAD51* genes of yeast and mammals [8,9]. The *RAD51* protein plays a critical role in repair of DNA double-strand breaks by homologous recombination in the yeast *Saccharomyces cerevisiea* [10], and is well conserved in higher eukaryotes [11–13]. The realization that *XRCC2* and *XRCC3* belong to the *RAD51* family of genes has fueled speculation that they also function in a pathway for DNA repair by homologous recombination.

Chromosome instability in the irs1 and irs1SF cell lines has been investigated by following the fate over many cell generations of human marker chromosomes introduced via microcell-mediated chromosome transfer. This method offers advantages over an assessment using only the endogenous chromosomes. Because a human marker chromosome can be painted with a human-specific fluorescent probe, any alteration to it can be readily seen against the background of hamster chromosomes. Breakage, deletion and translocation events involving the marker chromosome can be scored unambiguously. By following alterations of a marker chromosome over the expansion of multiple clonal cell populations, and applying a statistical analysis, it becomes possible to characterize chromosome instability quantitatively in a mutant cell line. The *XRCC2* and *XRCC3* loci have previously been mapped to human chromosomes 7q36 and 14q32.3 [3–5]. Chromosome transfer was used to introduce one copy of human chromosome 7 or 14 into irs1 and irs1SF mutant cells, respectively. Noncomplementing control chromosomes (4 and 7) were also transferred into irs1 and irs1SF cells. The results demonstrate that the *XRCC2* and *XRCC3* genes are essential for chromosome stability.

2. Materials and methods

2.1. Cell culture and microcell-mediated chromosome transfer

Human–hamster hybrid cells were grown in alpha-Modified Eagle's Medium (Gibco) with 10% fetal bovine serum, 100 units/ml of penicillin (Gibco) and 100 μg/ml of streptomycin (Gibco), 0.5 μg/ml Fungizone (Gibco), and 400 μg/ml G418 (Gibco). Cultures were maintained at 37°C in a humidified incubator with 5% carbon dioxide. Mouse A9 hybrid cell lines carrying single human chromosomes with a neomycin resistance marker (*NEO*) have been established and previously described [14]. Chromosome transfer was carried out according to the method of Kurimasa et al. [15]. Briefly, A9-human hybrid cells were treated with colcemid (0.05 μg/ml) for 48 h to form microcells, which were then harvested by centrifugation and filtration, and fused with irs1 or irs1SF cells using polyethylene glycol. G418 at 800 μg/ml was used to select for the *NEO* marker on transferred human chromosomes in hybrid cells. Hybrid clones of G418-resistant irs1 or irs1SF cells were isolated and transferred into T25 flasks for expansion. Each primary clone was screened by fluorescence in situ hybridization (FISH) of metaphase chromosome spreads for the presence of a single human chromosome. For passage of primary hybrid clones, confluent cultures were trypsinized and one tenth to one fifth of the cells were reseeded to a fresh T25 flask. For subcloning of primary clones, 100 cells were plated per 10 cm culture dish. After 10 to 14 days, secondary clones arose and were transferred individually to T25 flasks for expansion.

2.2. MMC exposure

Wild-type cell lines or complemented clones (V79, and irs1 with chromosome 7; AA8, and irs1SF with

chromosome 14), were plated for colony formation assays at 200 cells/10 cm dish, with MMC (Sigma, St. Louis, MO) at 0, 50 and 100 nM. Mutant cell lines or noncomplemented clones (irs1, or irs1 with chromosome 4; irs1SF, or irs1SF with chromosome 7) were plated at 200 and at 2000 cells/10 cm dish, with MMC at 0, 10 and 50 nM; at least 3 dishes per treatment. After ten to fourteen days, cell colonies were stained with 0.2% crystal violet in 70% ethanol. Colonies of fifty or more cells were counted.

2.3. Chromosome slide preparation

For chromosome harvest, cells were treated with 0.05 μg/ml colcemid (Gibco) for 1.5 h. The cells were then trypsinized, centrifuged and resuspended in hypotonic saline (0.075 M KCl) at 37°C for 12 min. The cells were fixed in a 3:1 mix of methanol and acetic acid and stored at −20°C. Fixed cell suspensions were transferred to glass slides and allowed to air-dry. For routine observations, chromosome slides were Giemsa stained.

2.4. Fluorescence in situ hybridization painting

After two weeks aging in air, chromosome slides were denatured in 70% formamide in 2 × SSC at 70°C for 2 min. Four single-strand Alu-repeat oligomers were synthesized as probes for human chromosomes:

A. GGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCCGA;
B. TCGGCCTCCCAAAGTGCTGGGATTACAGGCGTGAGCCACC;
C. GGAGGCTGAGGCAGGAGAATCGCTTGAACCCGGGAGGCGG;
D. CCGCCTCCCGGGTTCAAGCGATTCTCCTGCCTCAGCCTCC.

The four oligos were mixed in a 1:1:1:1 ratio. 0.5 μg/reaction of mixed probe were labeled with Cy3-dCTP (Amersham Life Science) using a terminal transferase reaction (Terminal Transferase Kit, Boehringer Mannheim). A synthetic single strand telomere-repeat oligomer $(TTAGGG)_7$ was labeled by the same method with Oregon green-dUTP (Molecular Probes, Eugene, OR, USA). A hybridization mixture comprising 0.3 μg/ml human Alu probe DNA and 0.25 μg/ml telomere probe DNA in 50% formamide, 2 × SSC, was applied to the slides. After overnight hybridization at 37°C, the slides were washed in 2 × SSC at 42°C three times, 15 min each, and then for 5 min in 1% Triton-X 100, phosphate buffer (pH 8.0). Chromosomes were counterstained with DAPI in antifade solution.

2.5. Statistical analysis

To quantitatively express quantitatively chromosomal change, the Chromosome Instability Index (CII) was applied. CII is defined as the mean number of unique rearrangements per cell in a population expanded from a single cell. CII for control and mutant cells (or, in the present case, complemented and noncomplemented mutant cells) are comparable only between clonal populations expanded for the same number of cell doublings. Statistical analysis and determination of clonal instability was done as follows. From all control (complemented) clones, an average CII ± standard error was calculated. Individual control clones are judged to be stable if their CII falls within a 98% confidence interval from the mean. No more than one clone in a hundred would be expected to exceed the upper boundary of this interval. By pooling data from stable control clones, a representative control CII was calculated. Each repair-deficient clone was then evaluated to determine whether it had a CII greater than the representative control CII, and if so the clone was labeled unstable. In performing this evaluation, an adjustment was made to the representative CII to account for the different lengths of the human marker chromosomes in the control and mutant hybrid cell lines. A 2 × 2 matrix (stable/unstable vs. control/mutant) was constructed. A chi-squared test was applied to determine whether there was a statistically significant difference in the proportions of stable to unstable clones between the control and mutant cell lines. A mutation was judged to induce chromosome instability only when the chi-squared test indicates a significant difference. A *t* test was also applied to compare the average CII between complemented and noncomplemented cell lines.

3. Results

3.1. Correction of MMC sensitivity and cloning efficiency in XRCC2- and XRCC3-complemented human–hamster hybrid cell clones

After microcell-mediated chromosome transfer, four groups of human–hamster hybrids, each carrying a single human chromosome, were isolated and confirmed by G-banding (data not shown). Clones of irs1 cells carrying human chromosome 7 (which contains the *XRCC2* gene) or human chromosome 4 (as a control) were designated irs1-C7 and irs1-C4 respectively. Clones of irs1SF cells carrying human chromosome 14 (which contains the *XRCC3* gene) or human chromosome 7 (as a control) were designated irs1SF-C14 and irs1SF-C7 respectively.

To determine whether appropriate human chromosomes could correct the mutant phenotypes of irs1 and irs1SF cells, several independent hybrids of each group were tested for resistance to MMC. Testing for complementation could also have been done by measuring resistance to ionizing radiation. However, while irs1 and irs1SF are only moderately sensitive to radiation, they are very highly sensitive to MMC. Recovery of MMC resistance therefore is a more sensitive test of complementation.

MMC sensitivity was measured in four irs1-C7 clones and three irs1-C4 clones, and these were compared to the sensitivities of irs1, and to V79, the parental cell line from which the irs1 mutant was derived. The relative sensitivities of the four cell types to MMC are shown in Fig. 1. The irs1-C4 clones were no more resistant to MMC than irs1 itself. The irs1-C7 clones recovered resistance to levels that appear slightly higher than those of V79, but no statistical significance can be attached to the difference. The irs1 and irs1SF cell lines also show reduced cloning efficiency relative to the parental lines from which they were derived. Cloning efficiencies (C.E.) were therefore measured as an additional indicator of complementation. The C.E. for irs1-C7 was 0.79 ± 0.02 (average $\pm$ standard error of 4 clones); somewhat lower than for wild-type V79 (0.92 ± 0.02). Curiously, the C.E. for irs1-C4 was only 0.28 ± 0.04 (average of 3 clones); even lower than for irs1 (0.50 ± 0.01).

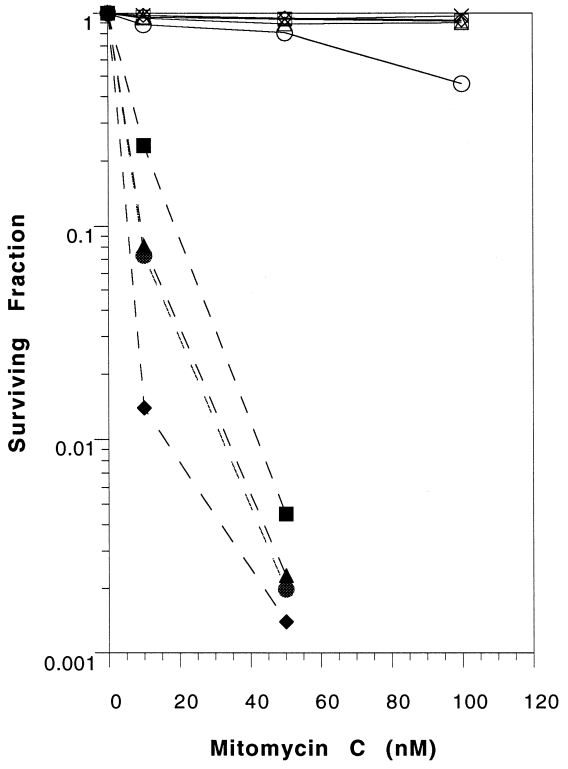

Fig. 1. MMC sensitivity of *XRCC2* complemented and noncomplemented cell clones. Four clones with human chromosome 7, irs1-C7#4 (□), irs1-C7#5 (◇), irs1-C7#7 (△) and irs1-C7#8 (×) showed resistance to MMC close to that of the wild type V79 (○), while three clones with human chromosome 4, irs1-C4#3 (●), irs1-C4#4 (▲), and irs1-C4#6 (◆) retained a sensitivity to MMC close to that of the mutant parent irs1 (■).

Similarly, MMC sensitivity and C.E. were measured in five irs1SF-C14 clones and four irs1SF-C7 clones, and these were compared to the sensitivity of irs1SF and of AA8, the parental cell line from which the irs1SF mutant was derived. Relative MMC sensitivities are shown in Fig. 2. The irs1SF-C7 clones were not consistently more resistant than irs1SF itself. The irs1SF-C14 clones substantially recovered MMC resistance, though not fully to the level of parental AA8 cells. The incomplete complementation seen here by chromosome transfer is similar to that reported previously for complementation by cDNA expression [3,9]. Incomplete complementation in this instance cannot be attributed to the absence of regulatory elements, since a complete chromosomal locus was transferred. The C.E. of irs1SF-C14 ($0.68 \pm$

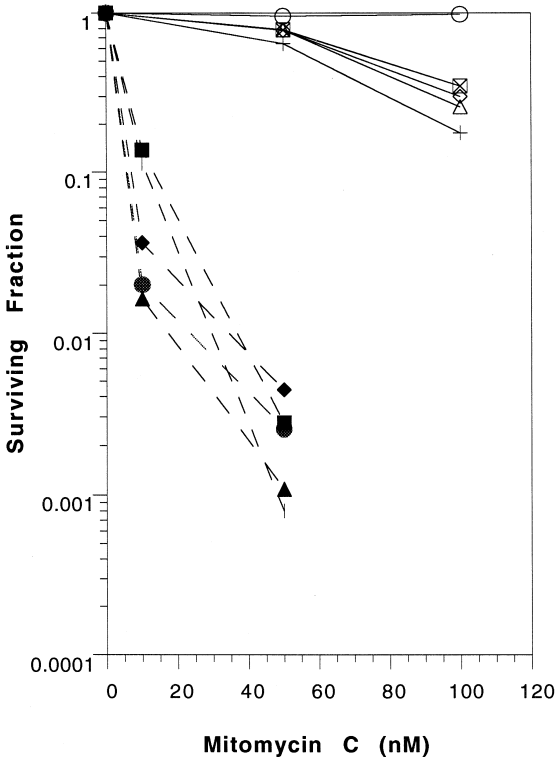

Fig. 2. MMC sensitivity of *XRCC3* complemented and noncomplemented cell clones. Five clones with human chromosome 14, irs1SF-C14#1 (□), irs1SF-C14#2 (◇), irs1SF-C14#3 (×), irs1SF-C14#6 (+), and irs1SF-C14#7 (△) showed increased resistance to MMC, though less than that of the wild type AA8 (○), while four clones with human chromosome 7, irs1SF-C7#1 (●), irs1SF-C7#2 (■), irs1SF-C7#3 (◆) and irs1SF-C7#4 (▲) retained a sensitivity to MMC close to that of the mutant parent irs1SF (|).

0.02; average of 5 clones) was nearly that of AA8 cells (0.74 ± 0.02). The C.E. of irs1SF-C7 was much lower (0.28 ± 0.02; average of 4 clones) and very similar to that of irs1SF (0.22 ± 0.01).

Thus human chromosomes 7 and 14, carrying the *XRCC2* and *XRCC3* repair genes respectively, are able to complement the MMC sensitivity of irs1 and irs1SF mutant cells (at least partially) and increase C.E. to near wild type levels.

3.2. Correction of chromosome stability in XRCC2- and XRCC3-complemented cells

The stability of the human marker chromosome was assessed in ten irs1-C7 clones, eight irs1-C4 clones, nine irs1SF-C14 clones and eight irs1SF-C7 clones. After chromosome transfer, individual hybrid colonies were expanded to a confluent T25 culture flask; estimated to represent at least twenty cell generations. FISH painting with a human Alu DNA probe was used to identify the single human chromosome against a background of hamster chromosomes (Fig. 3). Chromosome aberrations involving the human marker chromosome were scored in 100 metaphase cells for each clone. It should be noted that all hybrid cell lines in this study were maintained under G418 selection for the *NEO* gene on the human marker chromosomes. Chromosome rearrangements that result in loss of the *NEO* gene could not be transmitted under these conditions, and hence were not scored.

The results for irs1-C7 (*XRCC2*-complemented) and irs1-C4 (noncomplemented) clones are presented in Table 1. In irs1-C7 clones, only 13 cells with rearrangements of the human chromosome were found among the 1000 metaphase cells examined; a frequency of 1.3%. In contrast, irs1-C4 clones showed rearrangements of the human chromosome at a frequency of 39.5%, about thirty-fold higher. Several types of chromosome rearrangements were seen in irs1-C4 cells, including breaks, deletions (including terminal and interstitial deletions), balanced translocations (trans I), unbalanced translocations (trans II) and insertions (portions of the human chromosome inserted into hamster chromosomes). Chromosome 'gaps' cannot be reliably resolved by fluorescence microscopy, and so were not scored.

The average CII of irs1-C7 (complemented) clones was 0.013 ± 0.003. For irs1-C4 (noncomplemented) clones, mean CII was 0.325 ± 0.094, about twenty-one times higher when adjusted for different lengths of the human marker chromosome. By t test, the difference is significant to a level of $p < 0.005$. The CII for each of the irs1-C7 clones was then individually compared to the mean for the group (control mean). None of the ten clones exceeded the mean by more than 2.3 standard deviations (the upper boundary of a 98% confidence interval), and hence all were considered to be stable. CII for individual irs1-C4 clones were then compared to the control mean. All eight irs1-C4 clones exceeded the mean by more than 2.3 standard deviations, and so were judged unstable. A chi-square test was used to com-

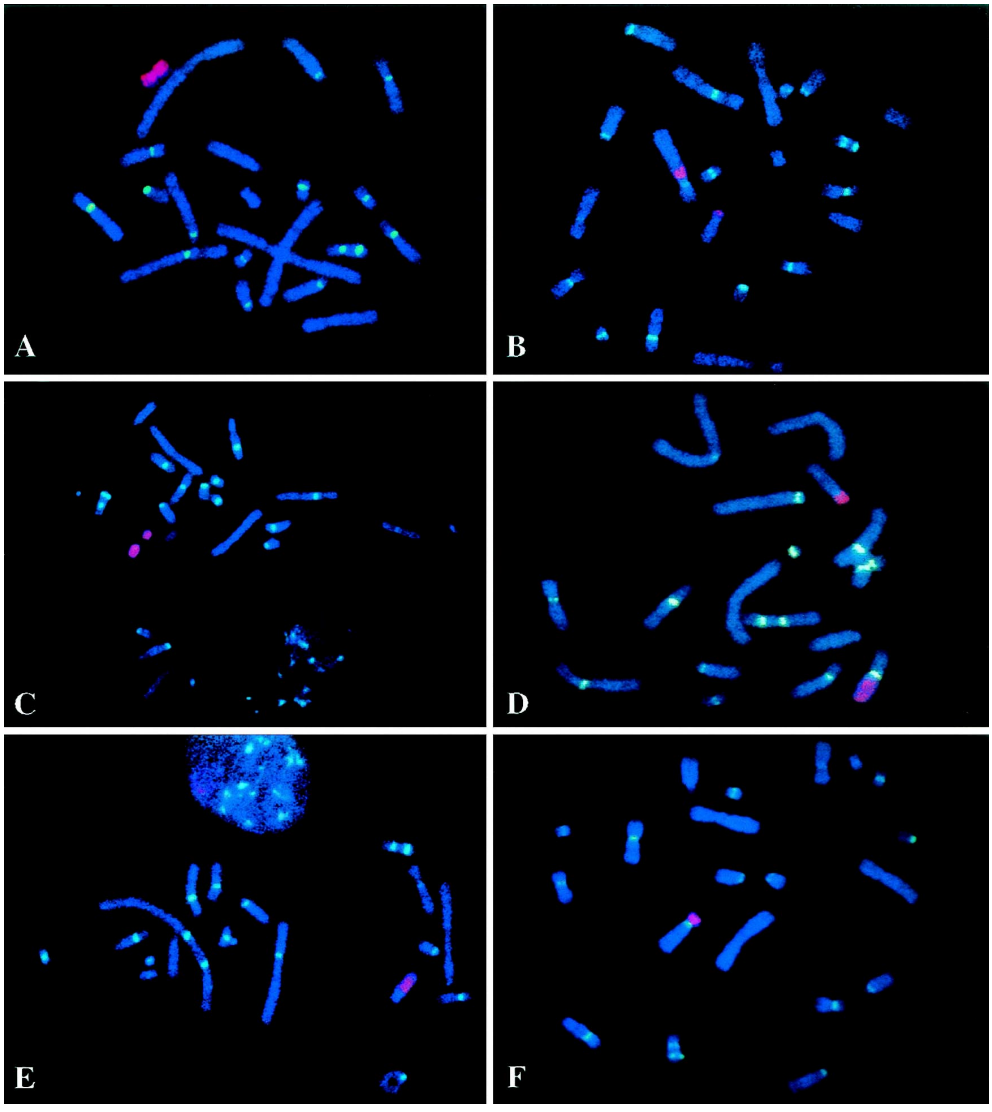

Fig. 3. Rearrangement of the human marker chromosome in human–hamster hybrid cells after FISH. The human chromosome has been hybridized with a human Alu probe (red). Hamster chromosomes appear blue. ITB (interstitial telomere band) are visualized using a mammalian telomeric probe $(TTAGGG)_7$ (green). A to F show different types of rearrangements that occurred in the human marker chromosome in noncomplemented *XRCC2* or *XRCC3* mutant cells: A. normal human chromosome 7 in an irs1SF-C7 cell; B. a fragment of human chromosome 7 inserted into a hamster chromosome in an irs1SF-C7 cell; C. a break in human chromosome 7 in an irs1SF-C7 cell; D. a balanced translocation with an ITB proximal to the junction site in an irs1SF-C7 cell; E. an unbalanced translocation in an irs1-C4 cell; F. an unbalanced translocation with an ITB proximal to the junction site in an irs1SF-C7 cell.

pare the irs1-C7 and irs1-C4 clones in aggregate. A significantly greater proportion of irs1-C4 clones were unstable than of irs1-C7 clones ($p < 0.001$).

A similar trend is apparent when irs1SF-C14 (*XRCC3*-complemented) clones and irs1SF-C7 (non-complemented) clones are compared (Table 2). Among 900 irs1SF-C14 metaphases, only 3 were found with rearrangements of the human chromosome, a frequency of 0.33%. For irs1SF-C7 clones, the frequency of metaphases with a rearranged hu-

Table 1
Chromosome rearrangements among irs1-C7 (*XRCC2*-complemented) and irs1-C4 (noncomplemented) primary clones

Primary clones	Metaphases scored	Metaphases with aberrations (%)[a]	Chromosome instability index[b]	Type of chromosomal aberrations[c]					
				Deletion[d]	Break[e]	TransI[f]	TransII[g]	Insertion	Others
irs1-C7									
#1	100	1	0.01	1	0	0	0	0	0
#2	100	2	0.02	2	0	0	0	0	0
#3	100	2	0.02	2	0	0	0	0	0
#4	100	0	0.00	0	0	0	0	0	0
#5	100	1	0.01	0	0	1	0	0	0
#6	100	1	0.01	0	0	0	1	0	0
#7	100	3	0.03	2	0	0	0	0	1
#8	100	1	0.01	1	0	0	0	0	0
#9	100	2	0.02	1	0	1	0	0	0
#10	100	0	0.00	0	0	0	0	0	0
irs1-C4									
#1	100	77	0.77	21	16	39	1	0	0
#2	100	53	0.53	22	0	24	0	0	7
#3	100	5	0.05	0	0	4	1	0	0
#4	100	24	0.22	3	1	4	13/15	0	1
#5	100	59	0.57	5	1	5/7	39	6	1
#6	100	12	0.12	4	0	7	0	1	0
#7	100	78	0.27	4	1	7/46	12/24	2	1
#8	100	8	0.07	4	0	1/2	2	0	0

[a] Only rearrangements of the human chromosome were scored.
[b] CII, the average number of unique rearrangements of the human chromosome within a clone.
[c] Unique rearrangements and total rearrangements scored. For example, '39' (irs1-C4#1 transI) means 39 unique rearrangements; '13/15' (irs1-C4#4 transII) means that 13 of total 15 transII are unique.
[d] Including terminal and interstitial deletions.
[e] Not including gaps.
[f] Balanced translocation.
[g] Unbalanced translocation.

man chromosome was 30.9%, about 93-fold higher. The average CII for irs1SF-C7 (noncomplemented) clones was 0.264 ± 0.064, about 50-fold higher than for irs1SF-C14 (complemented) clones (0.003 ± 0.002). By *t* test, the difference is significant to a level of $p < 0.001$. When the CII of individual irs1SF-C14 clones were compared to the mean for the group (control mean), all were found to differ by less than 2.3 standard deviations. However, all eight of the irs1SF-C7 clones differed by more than 2.3 standard deviations from the control mean and are hence unstable. By a chi-square test, the difference in proportions of unstable clones among the noncomplemented versus complemented clones is significant to a level of $p < 0.001$.

To further assess stability of the human marker chromosome in *XRCC3*-complemented and noncomplemented cells, one clone of each was subcloned. Subclones were expanded from single cells to a confluent T25 flask; roughly another twenty cell divisions. Table 3 shows results for twenty subclones expanded from primary clone irs1SF-C14 #1, and eighteen subclones expanded from primary clone irs1SF-C7 #1. In the irs1SF-C7 (noncomplemented) subclones, 1052 metaphases bearing an aberrant marker chromosome were found out of 1800, a frequency of 58.4%; markedly higher than the 18% seen in the parent primary clone irs1SF-C7 #1 (Table 2). Although some of the irs1SF-C7 subclones had lower CII than the primary clone from which

Table 2
Chromosome rearrangements among irs1SF-C14 (*XRCC3*-complemented) and irs1SF-C7 (noncomplemented) primary clones

Primary clones	Metaphases scored	Metaphases with aberrations (%)	Chromosome instability index	Type of chromosomal aberrations					
				Deletion	Break	TransI	TransII	Insertion	Others
irs1SF-C14									
#1	100	0	0.00	0	0	0	0	0	0
#2	100	0	0.00	0	0	0	0	0	0
#3	100	1	0.01	0	0	1	0	0	0
#4	100	0	0.00	0	0	0	0	0	0
#5	100	0	0.00	0	0	0	0	0	0
#6	100	2	0.02	0	0	2	0	0	0
#7	100	0	0.00	0	0	0	0	0	0
#8	100	0	0.00	0	0	0	0	0	0
#9	100	0	0.00	0	0	0	0	0	0
irs1SF-C7									
#1	100	18	0.07	1/8	1/4	2	3/4	0	0
#2	100	24	0.23	13	1	1	8/9	0	0
#3	100	63	0.63	31	13	0	10	8	1
#4	100	60	0.41	13/31	1	8	5/6	14	0
#5	100	20	0.20	10	1	2	4	3	0
#6	100	25	0.25	12	4	2	8	0	0
#7	100	26	0.22	8/9	4	4	2/5	2	2
#8	100	11	0.10	6	1	1	4/5	0	0

they were derived, the average CII of irs1SF-C7 subclones was 0.191 ± 0.032, increased from 0.070 for the primary clone. For the irs1SF-C14 (*XRCC3*-complemented) subclones, mean CII was 0.004 ± 0.001. A *t* test showed the mean CII of irs1SF-C7 subclones to be significantly higher than for irs1SF-C14 subclones ($p < 0.001$). None of the individual irs1SF-C14 subclones had CII differing from the control mean by more than 2.3 standard deviations. But among the eighteen irs1SF-C7 subclones, all eighteen had CII exceeding the control mean by more than 2.3 standard deviations. Again, by a chi-square test, the difference in proportions of unstable subclones among the noncomplemented versus complemented groups is significant to a level of $p < 0.001$.

Overall, human marker chromosomes were much more stable in *XRCC2*- and *XRCC3*-complemented clones than in noncomplemented mutant clones. The presence of *XRCC2* and *XRCC3* genes evidently stabilizes human chromosomes 7 and 14 in irs1-C7 and irs1SF-C14 cells. In the noncomplemented irs1-C4 and irs1SF-C7 cells, the absence of wild-type *XRCC2* or *XRCC3* genes is associated with sharply elevated frequencies of spontaneous aberrations in the human marker chromosome.

3.3. Transmissible chromosome aberrations occur in XRCC2- and XRCC3-deficient primary clones and subclones

The presence of persistent chromosome aberrations means that specific aberrations occurring in individual cells early in the expansion of a cell population have been transmitted to successive generations of daughter cells. This phenomenon is apparent in *XRCC2*- and *XRCC3*-deficient primary clones and *XRCC3*-deficient subclones, especially in the latter. For many of these clones, CII is not equal to the percentage of metaphases with aberrations involving the human marker chromosome. This is because metaphases containing the same aberrations are scored as a single event in determining CII. Persistent, or transmissible, chromosome aberrations were seen in four of eight *XRCC2*-deficient irs1-C4 primary clones examined (#4, #5, #7, and #8;

Table 1), and in five of eight *XRCC3*-deficient irs1SF-C7 primary clones (#1, #2, #4, #7 and #8; Table 2). Among subclones of irs1SF-C7#1, twelve of eighteen (subclones A, C, D, F, I, K, L, M, O, P, Q, and R; Table 3) also showed transmissible aberrations. The types of transmissible chromosome aberrations found were mainly deletions and unbalanced translocations. We have not seen persistent chromosome aberrations in the *XRCC2*- and *XRCC3*-complemented irs1-C7 or irs1SF-C14 primary clones or irs1SF-C14#1 subclones, indicating that the few chromosome aberrations seen in these populations

Table 3
Chromosome instability among irs1SF-C14 (*XRCC3*-complemented) subclones of and irs1SF-C7 (noncomplemented) subclones

Secondary clones	Metaphases scored	Metaphases with aberrations (%)	Chromosome instability index	Type of chromosomal aberrations					
				Deletion	Break	TransI	TransII	Insertion	Others
irs1SF-C14#1									
A	100	0	0.00	0	0	0	0	0	0
B	100	2	0.01	0	0	1/2	0	0	0
C	100	1	0.01	0	0	0	1	0	0
D	100	0	0.00	0	0	0	0	0	0
E	100	1	0.01	0	0	0	1	0	0
F	100	1	0.01	0	0	1	0	0	0
G	100	1	0.01	0	0	0	1	0	0
H	100	0	0.00	0	0	0	0	0	0
I	100	1	0.01	1	0	0	0	0	0
J	100	0	0.00	0	0	0	0	0	0
K	100	0	0.00	0	0	0	0	0	0
L	100	0	0.00	0	0	0	0	0	0
M	100	0	0.00	0	0	0	0	0	0
N	100	0	0.00	0	0	0	0	0	0
O	100	1	0.01	1	0	0	0	0	0
P	100	0	0.00	0	0	0	0	0	0
Q	100	0	0.00	0	0	0	0	0	0
R	100	1	0.01	1	0	0	0	0	0
S	100	0	0.00	0	0	0	0	0	0
T	100	0	0.00	0	0	0	0	0	0
irs1SF-C7#1									
A	100	100	0.03	0	0	0	3/100	0	0
B	100	46	0.46	13	14	6	12	1	0
C	100	100	0.02	0	0	0	1/99	1	0
D	100	99	0.03	1	0	0	1/97	1	0
E	100	11	0.11	7	0	1	4	0	0
F	100	93	0.20	3	0	6/13	10/72	1/2	0
G	100	18	0.18	8	0	5	7	0	0
H	100	13	0.13	2	1	5	5	0	0
I	100	98	0.05	0	0	0	5/98	0	0
J	100	21	0.21	6	6	2	7	0	0
K	100	31	0.28	8	1/4	10	9	0	0
L	100	33	0.32	16	2	1	10/11	1	2
M	100	48	0.46	20	4	4	18/20	0	0
N	100	24	0.24	12	3	3	5	1	0
O	100	22	0.20	9/10	0	1	10/11	0	0
P	100	100	0.09	0	0	0	7/98	1	1
Q	100	95	0.29	10	0	3	16/79	3	0
R	100	100	0.14	0	0	3	11/97	0	0

Table 4
Human chromosome rearrangements in *XRCC2*-complemented and noncomplemented cells during extended passage

Passage no.[a]	Metaphases scored	Metaphases with aberrations (%)	Type of chromosomal aberrations					
			Deletion	Break	TransI	TransII	Insertion	Others
irs1-C7#4								
P0	100	0	0	0	0	0	0	0
P3	100	4	1	0	2	1	0	0
P6	100	1	0	0	1	0	0	0
P9	100	2	0	0	0	0	0	2
irs1-C4#3								
P0	100	5	0	0	4	1	0	0
P3	100	13	2	0	2	9	0	0
P6	100	15	2	4	9	0	0	0
P9	100	20	2	2	6	8	1	0

[a]Approximately 3.3 cell divisions for each passage.

generally represent new chromosome changes that have not been transmitted.

3.4. Chromosome instability is transmissible during extended passage of XRCC2- and XRCC3-deficient clones

The results of the subcloning experiment described above showed that chromosome instability was transmissible in one clone of noncomplemented *XRCC3*-deficient cells. To more generally assess the persistence of chromosome instability in *XRCC2*- and *XRCC3*-deficient cells, one clone each of *XRCC2*- or *XRCC3*-complemented and noncomplemented cells was continuously subcultured for several weeks, with harvests for chromosome spreads at intervals. The results are shown in Tables 4 and 5. For *XRCC2*-deficient (clone irs1-C4 #3) cells, the frequency of chromosome aberrations increased from 5% at passage 0 to 20% at passage 9. Here, passage 0 means the primary colony expansion. Similarly in *XRCC3*-deficient cells (clone irs1SF-C7 #1), the frequency of aberrations increased from 18% at passage 0 to 65% at passage 9. In contrast, the human marker chromosomes in *XRCC2*- and *XRCC3*-complemented cells (irs1-C7 #7, irs1SF-C14 #1) were quite stable over extended passage.

Table 5
Human chromosome rearrangements in *XRCC3*-complemented and noncomplemented cells during extended passage

Passage no.[a]	Metaphases scored	Metaphases with aberrations (%)	Type of chromosomal aberrations					
			Deletion	Breaks	TransI	TransII	Insertion	Others
irs1SF-C14#1								
P0	100	0	0	0	0	0	0	0
P3	100	1	0	0	1	0	0	0
P6	100	0	0	0	0	0	0	0
P9	100	0	0	0	0	0	0	0
irs1SF-C7#1								
P0	100	18	8	4	2	4	0	0
P3	100	47	22	4	4	20	0	0
P6	100	55	29	2	6	24	0	0
P9	100	65	29	0	5	29	1	1

[a]Approximately 3.3 cell divisions for each passage.

3.5. Interstitial telomere-like repeats may be preferentially involved in rejoining of chromosome breaks

It has been proposed previously that interstitially located telomere-like repeat sequences ('interstitial telomere bands', ITB) can act as 'hot spots' in the rejoining of chromosome breaks [16–18]. Using FISH with the telomeric repeat $(TTAGGG)_7$ as a probe, we have analyzed interchromosome rejoining events, in which a portion of a human marker chromosome became translocated onto or inserted into a hamster chromosome, for proximity to ITB. ITB signals occurred at subcentric regions as well as other intrachromosomal locations in the endogenous Chinese hamster chromosomes (Fig. 3). We found that interchromosome joints were often in close proximity to ITB (i.e., no separation between them could be resolved by fluorescence microscopy at 1000× magnification) in *XRCC2*- or *XRCC3*-complemented and in noncomplemented cells (Fig. 3). This was the case in four out of seven events (57%) in irs1-C7 cells, in 78 of 314 events (25%) in irs1-C4 cells, in four of nine events (44%) in irs1SF-C14 cells, and in 648 of 1100 events (59%) in irs1SF-C7 cells. The frequent proximity of joints to interstitial telomere-like repeats supports the idea that a rejoining mechanism using these sequences is frequently involved in healing chromosome breaks. However, neither *XRCC2* or *XRCC3* appear to be needed for rejoining events of this kind. Although translocations and insertions were far more frequent in noncomplemented irs1 and irs1SF cells than in complemented controls, the relative proportions of joints in close proximity to telomere-like repeats were not conspicuously different.

4. Discussion

4.1. The XRCC2 and XRCC3 genes are required for stable maintenance of an introduced human chromosome

It has previously been observed that the chromosomes of irs1 and irs1SF cells show elevated numbers of gaps, breaks and rearrangements relative to the respective parental cell lines V79 and AA8. In irs1 cells, Tucker et al. [7] reported a higher frequency of spontaneous breaks and exchanges, and further increases after treatment with gamma rays or MMC. Increased formation of ring chromosomes in irs1 cells after X-irradiation has been noted by Okayasu et al. [19]. Full or partial correction of the spontaneous chromosome instability in irs1 cells after transfection with cDNA or genomic clones of the *XRCC2* gene has been reported by Cartwright et al. [8] and by Liu et al. [9]. In irs1SF cells, an excess of both spontaneous and X-ray induced chromosome aberrations was noted in the original description of the cell line [2], and full correction of this defect after transfection with a cosmid clone of the genomic *XRCC3* locus was reported by Tebbs et al. [3].

In this clonal analysis of chromosomal instability in irs1 and irs1SF cells, three questions have been considered: (1) What features of the cytogenetic data best describe chromosomal instability? (2) Is a particular clone unstable? and (3) Do the mutations induce instability? In regard to the first question, we have evaluated chromosome instability on the basis of a CII, defined as the average number of unique rearrangements occurring during the expansion of a population from a single cell. This is a better measure of instability than total aberrations, since the latter contains a variable contribution from transmission of aberrations occurring earlier or later during the expansion. An often overlooked difficulty in regard to the second two questions is that all cell populations are unstable to some extent, in the sense that chromosome aberrations arise spontaneously even in repair-proficient controls. In the method of analysis used here, this complication has been explicitly taken into account both in the definition of clonal instability and in the criterion for classifying a mutation as an inducer of instability.

We have found that human chromosome 7 (which contains the *XRCC2* gene) and human chromosome 14 (which contains the *XRCC3* gene) complemented irs1 and irs1SF cells for chromosome stability in primary clones and during extended passage. Among noncomplemented irs1 and irs1 SF clones, we found significantly higher mean CII; about 21-fold higher than complemented controls for irs1 and about 50-fold higher for irs1SF, when adjusted for the differences in marker chromosome lengths. Every individual noncomplemented primary clone was unstable by our statistical criteria and, in aggregate, the noncom-

plemented clones were more unstable than complemented controls with a very high degree of statistical certainty. Persistent chromosome rearrangements were seen in many of the noncomplemented primary clones. Subcloning and extended passage confirmed that chromosome instability is transmissible in noncomplemented clones of irs1 and irs1SF.

The phenotypes of noncomplemented irs1 and irsSF clones are very similar, but analysis of the data in Tables 1 and 2 indicates that they are not identical. There is a statistically significant difference ($p < 0.001$) in the proportions of balanced translocation and unbalanced translocation, with a greater proportion of balanced translocation in irs1-C4 clones, and of unbalanced translocation in irs1SF-C7 clones. There are also significantly more deletions in irs1SF-C7 clones ($p < 0.001$). While there was a tendency toward a higher proportion of breaks in irs1SF-C7 clones, the difference falls short of significance ($p \sim 0.06$) mainly due to an exceptionally large number of breaks in one irs1-C4 clone. Taken together, the cytogenetic data suggest a greater tendency for incomplete repair in the irs1SF-C7 mutant line. This implies that, while *XRCC2* and *XRCC3* genes probably act in the same DNA repair pathway, the role of *XRCC3* may be more critical than that of *XRCC2*. However, it is also possible that the more severe phenotype of the irs1SF mutant relative to irs1 is due to differences in genetic background between their parental cell lines V79 and AA8.

4.2. Chromosome instability in XRCC2- and XRCC3-deficient cells may be due to a defect in repair by homologous recombination associated with DNA replication

In yeast (*S. cerevisiea*), members of the *RAD51* gene family have been shown to play critical roles in repair of DNA double-strand breaks (DSB) through homologous recombination (reviewed in Ref. [20]). This repair pathway is evidently most efficient during S and G2 phases of the cell cycle, when a sister chromatid is available as a template. In both the haploid and diploid states, yeast is more resistant to ionizing radiation during these parts of the cell cycle [20]. In the diploid state, where both a homologous chromosome and a sister chromatid are available as templates for repair, the sister chromatid is used preferentially [21]. Genes involved in homologous recombination are also required in yeast for chromosome stability. Null mutation of *RAD52* in diploid strains of yeast results in frequent loss of chromosomes, and the frequency of loss is dramatically increased by exposure to X-rays, such that chromosome number in survivors may fall to near-haploid levels [22].

Because of their DNA sequence similarity to *S. cerevisiea RAD51*, mammalian *RAD51*-family genes have been proposed to participate in analogous pathways for repair of DSB though homologous recombination [8,9,12]. Chromosome instability has now been correlated with deficiency in three of the mammalian *RAD51*-family genes: *XRCC2* and *XRCC3* (as discussed above), and *RAD51* itself. In mouse, null mutation of *RAD51* by gene targeting results in early embryonic death, and is apparently lethal at the cellular level [23,24]. In early *RAD51*$^{-/-}$ embryos, growth is severely impaired and the few metaphase cells that can be recovered have sharply reduced chromosome numbers [24]. In a conditional gene knockout in DT40 chicken lymphoblastoid cells, shutdown of *RAD51* expression resulted in arrest of most of the population in G2/M phase of the cell cycle, with numerous chromosome breaks, followed by massive cell death [11]. The defect in chromosome stability arising from *RAD51* knockout is clearly more severe than for the *XRCC2* and *XRCC3* mutations in irs1 and irs1SF (since the latter are viable).

One possible explanation for the phenotypes of *RAD51*, *XRCC2* and *XRCC3* mutations is an essential function for homologous recombination in higher eukaryotes, in a form of repair that is closely coupled to replication and required for its successful completion. That mammalian cells cannot survive without Rad51, even though they are extremely proficient in DSB repair by nonhomologous end-joining [25], implies that the critical function of Rad51 is something more than DSB repair per se. The *XRCC2*- and *XRCC3*-mutant cell lines irs1 and irs1SF show only moderate hypersensitivity to ionizing radiation and no measurable defect in post-irradiation DSB repair [1,2], but nonetheless suffer reduced cloning efficiency and chromosome instability even in the absence of exogenous genomic insults. The irs1 and irs1SF cell lines also show sensitivity to UV, alkylat-

ing agents and especially crosslinking agents [1,2], all of which produce covalent DNA modifications that are barriers to replication [26]. Single-strand damage or breaks have been proposed to require a form of homologous recombination if they are not otherwise repaired before the passage of a replication fork [27,28]. A deficiency in such homologous replication-coupled repair may result in double-strand chromatid breaks, which in turn result in deletions or translocations when repaired nonhomologously. It should be noted that in the irs1 and irs1SF hamster–human hybrids reported here, only one copy of a human marker chromosome was transferred; no homologous chromosome is present. If homologous repair of the human marker chromosome takes place, this could only happen in S/G2 phases of the cell cycle using a sister chromatid as template.

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References

[1] N.J. Jones, R. Cox, J. Thacker, Isolation and cross-sensitivity of X-ray-sensitive mutants of V79-4 hamster cells, Mutat. Res. 183 (1987) 279–286.
[2] L.F. Fuller, R.B. Painter, A Chinese hamster ovary cell line hypersensitive to ionizing radiation and deficient in repair replication, Mutat. Res. 193 (1988) 109–121.
[3] R.S. Tebbs, Y. Zhao, J.D. Tucker, J.B. Scheerer, M.J. Siciliano, M. Hwang, N. Liu, R.J. Legerski, L.H. Thompson, Correction of chromosomal instability and sensitivity to diverse mutagens by a cloned cDNA of the XRCC3 DNA repair gene, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 6354–6358.
[4] J. Thacker, C.E. Tambini, P.J. Simpson, L.C. Tsui, S.W. Scherer, Localization to chromosome 7q36.1 of the human XRCC2 gene, determining sensitivity to DNA-damaging agents, Hum. Mol. Genet. 4 (1995) 113–120.
[5] N.J. Jones, Y. Zhao, M.J. Siciliano, L.H. Thompson, Assignment of the XRCC2 human DNA repair gene to chromosome 7q36 by complementation analysis, Genomics 26 (1995) 619–622.
[6] K. Caldecott, P. Jeggo, Cross-sensitivity of gamma-ray-sensitive hamster mutants to cross-linking agents, Mutat. Res. 255 (1991) 111–121.
[7] J.D. Tucker, N.J. Jones, N.A. Allen, J.L. Minkler, L.H. Thompson, A.V. Carrano, Cytogenetic characterization of the ionizing radiation-sensitive Chinese hamster mutant irs1, Mutat. Res. 254 (1991) 143–152.
[8] R. Cartwright, C.E. Tambini, P.J. Simpson, J. Thacker, The XRCC2 DNA repair gene from human and mouse encodes a novel member of the recA/RAD51 family, Nucleic Acids Res. 26 (1998) 3084–3089.
[9] N. Liu, J.E. Lamerdin, R.S. Tebbs, D. Schild, J.D. Tucker, M.R. Shen, K.W. Brookman, M.J. Siciliano, C.A. Walter, W. Fan, L.S. Narayana, Z.Q. Zhou, A.W. Adamson, K.J. Sorensen, D.J. Chen, N.J. Jones, L.H. Thompson, XRCC2 and XRCC3, new human Rad51-family members, promote chromosome stability and protect against DNA cross-links and other damages, Mol. Cell 1 (1998) 783–793.
[10] T.D. Petes, R.E. Malone, L.S. Symington, Recombination in yeast, in: J.R. Broach, J.R. Pringle, E.W. Jones (Eds.), The Molecular and Cellular Biology of the Yeast *Saccharomyces cerevisiae*, Cold Spring Harbor Laboratory Press, Plainview, NY, 1991, pp. 407–522.
[11] E. Sonoda, M.S. Sasaki, J.M. Buerstedde, O. Bezzubova, A. Shinohara, H. Ogawa, M. Takata, Y. Yamaguchi-Iwai, S. Takeda, Rad51-deficient vertebrate cells accumulate chromosomal breaks prior to cell death, Embo. J. 17 (1998) 598–608.
[12] P. Baumann, S.C. West, Role of the human RAD51 protein in homologous recombination and double-stranded-break repair, Trends Biochem. Sci. 23 (1998) 247–251.
[13] S. Vispe, C. Cazaux, C. Lesca, M. Defais, Overexpression of Rad51 protein stimulates homologous recombination and increases resistance of mammalian cells to ionizing radiation, Nucleic Acids Res. 26 (1998) 2859–2864.
[14] M. Koi, M. Shimizu, H. Morita, H. Yamada, M. Oshimura, Construction of mouse A9 clones containing a single human chromosome tagged with neomycin-resistance gene via microcell fusion, Jpn. J. Cancer Res. 80 (1989) 413–418.
[15] A. Kurimasa, K. Ohno, M. Oshimura, Restoration of the cholesterol metabolism in 3T3 cell lines derived from the sphingomyelinosis mouse (spm/spm) by transfer of a human chromosome 18, Hum. Genet. 92 (1993) 157–162.
[16] T. Ashley, J. Lieman, D.C. Ward, Multicolor FISH with a telomere repeat and Sry sequences shows that Sxr (sex reversal) in the mouse is a new type of chromosome rearrangement, Cytogenet. Cell Genet. 71 (1995) 217–222.
[17] J.P. Day, C.L. Limoli, W.F. Morgan, Recombination involving interstitial telomere repeat-like sequences promotes chromosomal instability in Chinese hamster cells, Carcinogenesis 19 (1998) 259–265.
[18] P. Slijepcevic, M.P. Hande, S.D. Bouffler, P. Lansdorp, P.E. Bryant, Telomere length, chromatin structure and chromosome fusigenic potential, Chromosoma 106 (1997) 413–421.

[19] R. Okayasu, G.E. Pantelias, G. Iliakis, Increased frequency of formation of interphase ring-chromosomes in radiosensitive irs-1 cells exposed to X-rays, Mutat. Res. 294 (1993) 199–206.
[20] J.C. Game, DNA double-strand breaks and the RAD50–RAD57 genes in *Saccharomyces*, Semin. Cancer Biol. 4 (1993) 73–83.
[21] L.C. Kadyk, L.H. Hartwell, Sister chromatids are preferred over homologs as substrates for recombinational repair in *Saccharomyces cerevisiae*, Genetics 132 (1992) 387–402.
[22] R.K. Mortimer, R. Contopoulou, D. Schild, Mitotic chromosome loss in a radiation-sensitive strain of the yeast *Saccharomyces cerevisiae*, Proc. Natl. Acad. Sci. U.S.A. 78 (1981) 5778–5782.
[23] T. Tsuzuki, Y. Fujii, K. Sakumi, Y. Tominaga, K. Nakao, M. Sekiguchi, A. Matsushiro, Y. Yoshimura, T. Morita, Targeted disruption of the Rad51 gene leads to lethality in embryonic mice, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 6236–6240.
[24] D.S. Lim, P. Hasty, A mutation in mouse rad51 results in an early embryonic lethal that is suppressed by a mutation in p53, Mol. Cell Biol. 16 (1996) 7133–7143.
[25] D. Roth, J. Wilson, Illegitimate recombination in mammalian cells, genetic recombination, in: R. Kucherlapati, G.R. Smith (Eds.), American Society for Microbiology, Washington, DC, 1988, pp. 621–653.
[26] E.C. Friedberg, G.C. Walker, W. Siede, DNA repair and mutagenesis, American Society for Microbiology, Washington, DC, 1995.
[27] N.P. Higgins, K. Kato, B. Strauss, A model for replication repair in mammalian cells, J. Mol. Biol. 101 (1976) 417–425.
[28] P. Howard-Flanders, S.C. West, A. Stasiak, Role of RecA protein spiral filaments in genetic recombination, Nature 309 (1984) 215–219.