Radiation-induced Genomic Rearrangements Formed by Nonhomologous End-Joining of DNA Double-Strand Breaks

Kai Rothkamm, Martin Kühne, Penny A. Jeggo, and Markus Löbrich

Fachrichtung Biophysik, Universität des Saarlandes, D-66421 Homburg/Saar, Germany. [K. R., M. K., M. L. J., and MRC Cell Mutation Unit, University of Sussex, Brighton BN1 9RR, United Kingdom [P. A. J.]

ABSTRACT

Two major pathways for repairing DNA double-strand breaks (DSBs) have been identified in mammalian cells, nonhomologous end-joining (NHEJ) and homologous recombination (HR). Inactivation of NHEJ is known to lead to an elevated level of spontaneous and radiation-induced chromosomal rearrangements associated with an increased risk of tumorigenesis. This has raised the idea of a caretaker role for NHEJ. It is, however, not known whether NHEJ itself can also cause rearrangements. To investigate, on the DNA level, the influence of a defect in NHEJ on the formation of genomic rearrangements, we applied an assay based on Southern hybridization that allows the identification and quantification of incorrectly rejoined DSBs. These repair events are absent in wild-type cells but revealed a misrejoining frequency of 10% in NHEJ-deficient cells. This shows that in situations of separated breaks, NHEJ deficiency leads to genomic rearrangements, in agreement with chromosomal studies. However, if multiple DSBs coincide, even wild-type cells form genomic rearrangements frequently. These repair events are absent in Ku80-, DNA-PKcs-, and DNA ligase IV-deficient cells. Low-dose-rate experiments, in which the cells were exposed to 80 Gy over a period of 14 days under repair conditions, led to no detectable misrejoining in wild-type cells but revealed a misrejoining frequency of 10% in NHEJ-deficient cells. This suggests that in situations of separated breaks, NHEJ deficiency leads to genomic rearrangements, in agreement with chromosomal studies. However, if multiple DSBs coincide, even wild-type cells form genomic rearrangements frequently. These repair events are absent in wild-type cells but revealed a misrejoining frequency of 10% in NHEJ-deficient cells. This strongly suggests that NHEJ has, in addition to its caretaker role, also the potential to effect genomic rearrangements. We propose that it serves as an efficient pathway for rejoining correct break ends in situations of separated breaks but generates genomic rearrangements if DSBs are close in time and space.

INTRODUCTION

DSBs in chromosomes can be produced endogenously during replication and meiosis and by exogenous agents such as ionizing radiation. They have the potential to be a most disruptive form of DNA damage; if left unrepaired, they may lead to loss of genetic information and cell death (1), and if repaired incorrectly, can lead to carcinogenesis through directly induced or delayed translocations, inversions, or deletions (2). It is of cardinal importance to understand the mechanisms and the factors involved in correct and incorrect DSB repair.

There are, at least, two independent pathways for repairing DSBs, NHEJ and HR. NHEJ is the predominant mechanism in mammalian cells and involves the DNA end-binding heterodimer Ku70/Ku80, the catalytic subunit of the DNA-PK, the XRCC4 gene product and DNA ligase IV (reviewed in Refs. 3–5). Cell lines with mutations in any of these genes are radiation sensitive and show marked deficiencies in DSB repair (6–14). In addition to controlling repair of DSBs induced by ionizing radiation, DNA-PK and its associated components are also required for the joining of DSBs that arise during V(D)J recombination of immunoglobulin and T-cell receptor genes (15). Recently, it has become clear that proteins of the NHEJ pathway also play an essential role during normal development and in maintaining genomic stability, which is required for suppression of tumorigenesis (16, 17). However, HR also plays a crucial role in DSB repair in vertebrate cells (18–22). The process has been extensively studied in yeast and involves proteins of the RAD52 epistasis group, which seem to be conserved from yeast to humans (reviewed in Refs. 23 and 24). In yeast, HR is a high fidelity repair mechanism because it uses an undamaged template to retrieve any information lost at a break site. In higher organisms, it is possible that the fidelity of HR might be compromised if recombination occurs at non-allelic homologous sequences, such as repeat sequences (ectopic recombination; Refs. 25–27). Little is known about the fidelity of NHEJ in higher organisms. Studies with yeast suggest that NHEJ is usually accurate but that the rejoined ends are sometimes associated with small deletions and insertions (reviewed in Ref. 28). Investigations using mammalian cells also indicate that this mechanism does not involve significant regions of homology because only 1–4 bp of overlap are usually observed at the break site (29). However, these studies have been carried out with restriction enzyme-cut breaks, whereas limited information is available about the mechanisms and the enzymatic pathways involved in rejoining radiation-induced breaks. A challenge ahead is the evaluation of the fidelity of DSB rejoining in higher organisms and the contribution of NHEJ or HR to the formation of genomic rearrangements. Here, we use a method that allows the investigation of rejoining involving genomic rearrangements and thereby identify some genetic factors mediating this process.

MATERIALS AND METHODS

Cell Lines. CHO cells (CHO-K1, CHO-AA8, xrs-6, and V3) and primary human fibroblasts (MRC-5 and 180BR) were grown in MEM, mouse embryonic fibroblasts (C.B-17, scid, and RAD54+/−) in DMEM, both supplemented with 10% (20% for 180BR) FCS and antibiotics. All incubations were performed at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Nondividing cultures, obtained by growing cells to superconfluency, were used in all experiments (at least 90% in G1, as determined by flow cytometry).

Irradiation and Repair Incubation. For acute exposure experiments, X-irradiation was performed at 80 kV and 30 mA at a dose rate of ~23 Gy/min as determined by chemical dosimetry. Confluent cells were irradiated in 75-cm2 culture flasks filled with 5 ml of ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, and 1.5 mM KH2PO4, pH 7.45). For repair incubation, PBS was replaced with the original medium. For LDR experiments, confluent MRC-5 and 180BR cells were exposed inside an incubator (which was placed in a γ-irradiation room) to 80 Gy 60Co γ-rays over 14 days (5.7 Gy/day; the distance from the 60Co source to the incubator containing the cells was ~2.5 m) under repair conditions, i.e., in growth medium at 37°C. Preliminary control experiments had shown that confluent primary human fibroblasts irradiated with 80 Gy of X-rays remain as an attached monolayer without any sign of DNA degradation for at least up to 6 weeks if the medium is changed regularly every 7–10 days. Therefore, the LDR experiments were performed with confluent cells placed in a γ-irradiation room.
introduced after ~7 days for about 1–2 h, and the medium was changed. Cell counting was performed for all repair samples, ensuring that no cell loss had occurred during the incubation period. After irradiation and repair incubation, cells were harvested, embedded in agarose plugs, and lysed without subsequent RNase treatment.

**Conventional PFGE for Total DSB Rejoining Measurements.** To determine total DSB rejoining, DNA was separated by PFGE without prior restriction enzyme digestion. Electrophoresis was carried out with a CHEF DR III system in agarose gels. The gels were run at 14°C with linearly increasing pulse times from 50 to 5000 s over 65 h at a field strength of 1.5 V/cm. Gels were stained with ethidium bromide and photographed with a digital camera system under UV transillumination. Quantitative analysis was performed with commercially available software. The fraction of DNA below an exclusion size of 2.2 Mbp as determined from the largest chromosome of *Saccharomyces cerevisiae* was quantified. Experiments measuring the fraction of DNA below 2.2 Mbp as a function of dose were performed in parallel with repair experiments, and the results served as calibration to obtain relative numbers of remaining DSBs from the fraction of DNA <2.2 Mbp in the repair samples (according to Rydberg et al. [30]).

**Hybridization Assay for Correct DSB Rejoining Measurements.** For determining correct DSB rejoining, DNA was digested with restriction enzymes prior to electrophoresis. *Not*I was used for DNA of human cells, *Mlu*I for DNA of hamster cells, and *Sph*I for DNA of mouse cells. The gels were run for 115 h at 1.5 V/cm with pulse times from 500 to 3000 s (for optimal separation of human *Not*I fragments) or for 46 h at 3 V/cm with 40–800 s (for separating the rodent *Mlu*I and *Sph*I fragments). After PFGE separation, the DNA was partly depurinated and vacuum-blotted onto a charged nylon membrane by alkaline transfer. Membranes were prehybridized for at least 4 h at 65°C, followed by hybridization for 15–20 h at 65°C. The DNA probe *D21Sl4* hybridizes to a 3.2-Mbp *Not*I restriction fragment of human DNA, the dihydrofolate reductase cDNA probe to a 1.9-Mbp *Mlu*I fragment of hamster DNA, and the DNA probe *Fre2* to a 1.3-Mbp *Sph*I fragment of mouse DNA. Probes were labeled by random priming with [*α-32P]*dCTP. Filters were wrapped in saran wrap after washing and exposed overnight to an imaging screen, and the screen was scanned by a Fuji BAS 1000 phosphorimager. Quantitative analysis was carried out using the phosphorimaging software. The conventional PFGE and the hybridization assay were applied in parallel to all samples of an experiment. A detailed description of the assays, including a description of the evaluation procedure, are published in Rothkamm and Löbrich (31).

### RESULTS

**Genomic Rearrangements Are Formed by Joining Ends from Different DSBs.** Our previous work showed that the repair of DSBs can be investigated by measuring the reconstitution of a restriction fragment to its original size with an assay based on quantitative Southern hybridization (Refs. 31 and 32; see also Ref. 33). After exposure of primary G1-phase human fibroblasts (MRC-5) to ionizing radiation, the band intensity of a 3.2-Mbp genomic *Not*I restriction fragment decreases with increasing radiation dose, and a smear below the band accumulates (Fig. 1B, Lanes 1–6 and Fig. 1C), demonstrating the formation of DSBs within this fragment. After 24 h of repair incubation after 80 Gy of X-irradiation, the band was partly reconstituted, so that its intensity corresponded to the band intensity of a sample irradiated with half the dose and not incubated for repair (compare in Fig. 1B, Lane 5 with Lane 7, and see Fig. 1D). This indicates that 50% of the break ends are rejoined in a manner that reconstitutes the original restriction fragment. Results from a conventional PFGE assay that detects all rejoining events irrespective of whether or not the intact restriction fragment is reconstituted show nearly complete DSB repair after 24 h (Fig. 1A, compare Lanes 1 and 2 with Lane 7). Hence, after 80 Gy, ~50% of the repair events do not lead to reconstitution of the original restriction fragment and therefore represent misrepair events, which result in fragments smaller or larger than the original restriction fragment. We term this class of events, which includes, e.g., translocations or inversions, “rearrangement rejoining” or “DSB misrejoining” and contrast it to “correct DSB rejoining,” which describes restriction fragment reconstitution. The correct rejoining events include precise rejoining in which the original sequence is restored as well as imprecise DSB rejoining with gain or loss of short DNA sequences at the junctions leading to a restriction fragment that cannot be distinguished from the original fragment (the resolution of the assay is on the order of 100 kbp). Direct evidence that an appreciable percentage of the repair events lead to DSB misrejoining is provided in Fig. 1B (Lane 7) and in Fig. 1D by the appearance of a hybridization signal above the intact restriction fragment. The aberrant restriction fragments form a continuous distribu-
tion and only appear as a band in a size range that is not resolved electrophoretically (an electrophoretic compression zone in which fragments larger than approximately 4 or 6 Mbp, depending on the electrophoresis conditions, comigrate, as seen in Fig. 1, A and B).

Because DSB misrejoining likely requires an interaction between two breaks, the probability for misrejoining may be expected to depend on the number and proximity of the breaks. Indeed, we have shown recently that fractionating a given X-ray dose significantly decreases the DSB misrejoining frequency and increases the probability of joining correct, i.e., formerly connected, DSB ends (34). Here, we have γ-irradiated G1-phase MRC-5 cells at LDR such that a total of 80 Gy was delivered in 14 days. Because the cells were allowed to repair DSBs during LDR exposure, this procedure leads to a pronounced separation of DSBs in time and space which is expected to increase significantly the probability of correct break rejoining. In line with this expectation, we observed that continuous LDR irradiation results in very efficient overall rejoining as determined by the conventional PFGE assay (Fig. 1A, compare Lanes 9–12 with Lane 13) and also leads to complete restriction fragment reconstitution without any detectable sign of DSB misrejoining (Fig. 1B, compare Lanes 9–12 with Lane 13 and see Fig. 1E). This substantiates our suggestion that genomic rearrangements are formed by an interaction mechanism between DSBs in sufficient proximity. Moreover, because complete restriction fragment reconstitution is seen after 80 Gy LDR irradiation, our results show that rearrangement rejoining observed after an acute 80 Gy exposure cannot be attributed to an artifactual cellular response after a nonphysiological dose of 80 Gy but rather reflects a situation in which cells receive multiple DSBs at the same time. For a quantitative comparison, it may be helpful to consider that an acute X-ray dose of 80 Gy induces on average 1 DSB per 1.3 Mbp (all 4600 breaks per diploid genome are induced is helpful to consider that an acute X-ray dose of 80 Gy induces on average 1 DSB per 1.3 Mbp (all 4600 breaks per diploid genome are induced)

Because the time course for total and correct rejoining suggests that the slowly rejoined DSBs are likely to be those misrejoined because there is no change in the level of restriction fragment reconstitution during the period that this process operates. The time course for DSB misrejoining in MRC-5 cells as determined from the difference between total rejoining and correct rejoining is shown in Fig. 2D (□).

We next investigated the radiosensitive primary human fibroblast cell line, 180BR (36, 37), which carries a defect in DNA ligase IV (38), for its ability to join correct and incorrect, i.e., formerly unconnected, break ends in the same 3.2-Mbp NotI restriction fragment that was analyzed in MRC-5 cells. Conventional PFGE analysis revealed a pronounced DSB rejoining defect in these cells with a substantial fraction of DSBs remaining unrejoined 24 h after irradiation (Fig. 2A, Lane 10). Investigation of restriction fragment reconstitution by the hybridization approach showed slightly increasing band intensities with increasing incubation time (Fig. 2B, Lanes 5–10) such that the band intensity of a sample irradiated with 80 Gy and incubated 24 h for repair corresponded to the band intensity of a sample irradiated with 60 Gy and not repair incubated (Fig. 2E). The time course for total and correct rejoining in 180BR cells is shown in Fig. 2C. Both total rejoining (■ and correct rejoining (□ occur with slow kinetics for up to 24 h, and no significant difference between the two assays was detectable. The similarity in the time course suggests that DSB misrejoining, which is determined from the difference between total and correct rejoining, is greatly diminished in 180BR cells compared with MRC-5 cells (Fig. 2D, compare ■ and □). This is further substantiated by examining the restriction fragment size distribution in the 24-h repair sample (Fig. 2E), which shows that no fragments larger than the original restriction fragment are generated in 180BR cells, in strong contrast to the result with MRC-5 cells (Fig. 2B, compare Lanes 6–10 with Fig. 1B, Lane 7 and also Fig. 2E with Fig. 1D). We conclude that the vast majority of genomic rearrangements seen in wild-type primary human fibroblasts after an acute 80-Gy X-ray dose occur by a mechanism that is dependent on DNA ligase IV.

To investigate DNA ligase IV-deficient 180BR cells under conditions in which repair-proficient MRC-5 cells show complete restriction fragment reconstitution (and therefore no DSB misrejoining), we studied DSBR misrejoining in 180BR cells after 80 Gy of LDR γ-irradiation. If the same LDR protocol that was used for the experiment with MRC-5 cells (shown in Fig. 1E) is applied to DNA ligase IV-deficient cells, overall rejoining as determined by the conventional PFGE assay is essentially complete (Fig. 2A, compare Lanes 12 and 13 with Lane 15). However, restriction fragment reconstitution is not complete, and some DSB misrejoining can be detected by the occurrence of fragments larger than the original restriction fragment (Fig. 2B, compare Lanes 12 and 13 with Lane 15, and see Fig. 2F). An estimation of the level of DSB misrejoining derived from a series of such experiments with MRC-5 and 180BR cells exposed to LDR γ-irradiation is shown in Fig. 2G. Although MRC-5 cells do not form a measurable amount of genomic rearrangements, a DSB misrejoining frequency of ~10% is detectable in 180BR cells. Thus, in a situation...
when repair-proficient cells show no genomic rearrangements, a deficiency of DNA ligase IV leads to the appearance of rearrangements. DNA-PK Mediates the Formation of Genomic Rearrangements after High Radiation Doses. Because DNA ligase IV functions in NHEJ, we next asked whether other proteins involved in this process are required for the misrejoining events observed after an acute 80 Gy of X-irradiation. Total rejoining was measured by the conventional PFGE approach, which detects all rejoining events irrespective of whether the intact restriction fragment is reconstituted (closed symbols). Correct rejoining was measured with the hybridization assay (open symbols) in which the number of DSBs that have not been correctly rejoined can be quantified by determining the negative logarithm of the ratio of the band representing the original restriction fragment to the total hybridization signal of the lane, which represents all fragments containing the hybridization site and is a measure for the amount of DNA loaded per lane (see Refs. 31 and 32 for details). The profoundly different kinetics for total and correct rejoining in MRC-5 cells (-----) are indicative of DSB misrejoining. For 180BR cells, total and correct rejoining can be described by a single curve (-----), indicating that nearly all rejoining events lead to restriction fragment reconstitution. The data points are the average of two to three experiments. The curves are fitted by hand. D, fraction of the initially induced DSBs, which are misrejoined in MRC-5 and 180BR cells after various times after irradiation. The data are obtained from C from the difference between total and correct rejoining. Bars, the cumulative SE of the measurements for total and correct rejoining. E, intensity profiles of Lanes 4 and 10 of the Southern blot in B. After irradiation with an acute 80-Gy X-ray dose and repair incubation for 24 h, no fragments larger than the intact restriction fragment were formed. This supports the result in D that no misrejoining occurs in 180BR cells. F, intensity profiles of Lanes 13 and 15 of the Southern blot in B. Continuous γ-irradiation with 80 Gy over 14 days under repair conditions does not lead to complete restriction fragment reconstitution and reveals some DSB misrejoining in 180BR cells. G, fraction of the initially induced DSBs, which are misrejoined in MRC-5 and 180BR cells, continuously exposed with 80 Gy γ-rays over 14 days under repair conditions. DSB misrejoining is calculated from the difference between total rejoining and correct rejoining as determined in four independent experiments with duplicate samples. Bars, the cumulative SE.
fraction of DSBs remaining unrejoined. The time course for total and correct rejoining in xrs-6 and V3 cells is shown in Fig. 3A. Both total rejoining (–) and correct rejoining (●) continue with slow kinetics for up to 24 h, and no significant difference between the two assays is detectable. The similarity in the time course is indicative of a strongly diminished DSB misrejoining activity in these mutants compared with their parental cell lines (Fig. 3B). This is further supported by the absence of any fragments larger than the original restriction fragment in the repair incubated samples (Fig. 3C, bottom panels). We conclude that no DSB misrejoining is detectable in either V3 or xrs-6 cells (in striking contrast to a misrejoining frequency of 40–50% in CHO-K1 and CHO-AA8 cells). The same result was obtained using the SpII restriction fragment with DNA-PKcs-defective scid mouse fibroblasts (40, 41). In contrast to their wild-type counterpart C.B-17, no difference between the time course of total and correct rejoining was observed in scid cells (Fig. 4A, left panel and Fig. 4B), and no sign of DSB misrejoining was detectable on the Southern blot (Fig. 4C, middle panel). We conclude that the vast majority of genomic rearrangements seen in wild-type cells after an acute 80-Gy X-ray exposure occur by a DNA-PK-dependent end-joining pathway.

**Rad54 Is Dispensable for DSB Repair in the G1 Phase of the Cell Cycle.** We next analyzed the role of HR in joining correct and incorrect break ends. Because residual DSB rejoining by restriction fragment reconstitution was observed in DNA-PK-defective cell lines, we tested whether a DNA-PK/DNA ligase IV-independent repair pathway may mediate the rejoining of correct break ends and specifically asked whether Rad54 is involved in this process. Analysis of the SpII restriction fragment in RAD54−/− mouse embryonic fibroblasts in the G1 phase of the cell cycle revealed wild-type characteristics for both total and correct DSB rejoining (Fig. 4A, right panel). The band intensity of an 80-Gy repair sample is reconstituted within 2 h to the 50% level and does not increase further at longer repair times. Total rejoining includes a second slower component that proceeds for 24 h until DSB repair is essentially complete and gives rise to DSB misrejoining (Fig. 4B). DSB misrejoining is also visible by the appearance of fragments larger than the intact restriction fragment (Fig. 4C, right panel), in agreement with the results obtained in C.B-17 and CHO cells. We conclude that in G1 neither rejoining of correct break ends nor DSB misrejoining is significantly affected by a defect in homologous recombination.

**DISCUSSION**

**Formation of Genomic Rearrangements in Situations of Few or Multiple Breaks.** In this study, we show that DSB rejoining in mammalian cells can be classified into two categories: the rejoining of correct, i.e., formerly connected, break ends leading to restriction fragment reconstitution and the joining of ends from different DSBs, which generates genomic rearrangements. The probability with which a break end is either joined to a correct or an incorrect break end depends upon the spatial proximity of the DSBs. If no other DSBs are nearby at the time of repair, the broken ends are likely to be rejoined to their correct end. The presence of multiple DSBs at the same time enhances misrejoining in repair-proficient cells. To investigate the pathway mediating the formation of genomic rearrangements, it is necessary to choose conditions in which wild-type cells exhibit rearrangements by the appearance of aberrant restriction fragments. Such conditions necessitate relatively high radiation doses. However, under the conditions examined, the capacity for DSB rejoining is not saturated, and the kinetics for correct and incorrect rejoining are nearly identical for primary human and transformed hamster and mouse cells. Primary human fibroblasts do not undergo apoptosis after irradiation, demonstrating that misrejoining cannot be attributed to a
secondary effect relating to apoptosis. Moreover, 180BR cells, which show elevated radiosensitivity compared with MRC-5 cells, show reduced levels of misrejoining. Taken together, this provides strong evidence that our results represent the operation of a defined repair process. We show that after an acute exposure with 80 Gy of X-rays, the majority of genomic rearrangements depend upon the enzymes that function in NHEJ because DSB misrejoining, which occurs in wild-type cells within 24 h, is greatly diminished in cell lines deficient in Ku80, DNA-PKcs, or DNA ligase IV. Interestingly, DSB misrejoining after high-dose exposure of S. cerevisiae was also found to depend largely on Yku70, a component of the NHEJ pathway in yeast (42).

This result is particularly striking in view of the role of NHEJ as a caretaker of chromosomal integrity (16, 17) and appears to be contradictory with results from cytological studies that demonstrate an increased level of radiation-induced chromosomal exchanges in cells defective in DNA-PK-dependent end-joining (43–46). These studies indicate that, at the low doses used in cytological experiments (usually a few Gy), an alternative error-prone repair pathway compensates for the loss of DNA-PK-dependent end-joining and imply that NHEJ causes fewer rearrangements than this erroneous alternative repair pathway. Our studies involving 80 Gy of LDR γ-irradiation are likely to represent a situation comparable with the cytological studies carried out at low doses. In the LDR experiments, only very few breaks are induced at any given time, and because DSBs are repaired during irradiation, cells rarely encounter multiple breaks simultaneously. This is substantiated by complete overall rejoining in wild-type MRC-5 cells (Fig. 1A, Lanes 9–12) as well as in DNA ligase IV-deficient 180BR cells (Fig. 2A, Lanes 12 and 13). Such a situation leads to complete restriction fragment reconstitution without any detectable sign of DSB misrejoining in MRC-5 cells (Fig. 1, B and E, Lanes 9–12) but to some DSB misrejoining in 180BR cells (Fig. 2, F and G). Thus, in perfect agreement with results of cytological studies at low doses, LDR DSB repair experiments in NHEJ-deficient cells indicate that an alternative error-prone repair pathway compensates for NHEJ and generates genomic rearrangements that are not seen in wild-type cells. This is, to the best of our knowledge, the first time that the increased level of spontaneous and radiation-induced chromosomal exchanges observed in NHEJ-deficient cells (16, 17, 43–46) is shown to be reflected by an increase in the level of misrejoined genomic DSBs.

Despite this parallelism between chromosomal investigations and our LDR misrejoining experiments, however, these studies do not allow an evaluation of whether NHEJ per se can generate rearrangements. To evaluate this, we have had to use high acute doses of irradiation to enable an investigation of DSB repair in the presence of multiple breaks. Our results demonstrate that NHEJ can frequently mediate genomic rearrangements when DSBs occur in close proximity (the comparison between DSB misrejoining in the presence of few or multiple breaks is illustrated by the comparison between Fig. 2, G and D).

Although the error-prone nature of NHEJ is uncovered in the present study after high doses, we argue that the observed genomic rearrangements represent the potential of the NHEJ mechanism to rejoin breaks incorrectly and that such misrejoining events will also occur under physiological conditions when multiple DSBs happen to coincide within a critical interaction distance. This may occasionally be the case for DSBs produced by endogenous processes and might thereby contribute to tumorigenesis in normal individuals. The ratio of correct:incorrect rejoining may be particularly important after densely ionizing irradiation where multiple breaks occur in close proximity, even at very low doses (several DSBs are induced along the path of a single particle). In line with this, genomic rearrangement rejoining after α-particle exposure was observed recently to be dose independent (47). Because a large fraction of the average annual background exposure of the human population to ionizing radiation comes from densely ionizing α-particles generated during decays of radon and radon daughters (48), our findings are likely to have major implications for radiation protection issues.

**DSB Rejoining in the Absence of NHEJ.** Investigation of the rejoining kinetics displayed in Figs. 2C, 3A, and 4A reveals that total rejoining in repair-proficient cells includes a fast and a slow compo-

---

**Fig. 4.** DSB rejoining in repair-proficient (CB17), repair-deficient (scid), and RAD54−/− mouse embryonic fibroblasts. A, rejoining kinetics of DSBs in C.B-17 and DNA-PKcs-deficient scid cells (left panel) and in RAD54−/− cells after acute 80 Gy of X-irradiation. Total rejoining was measured by conventional PGE (closed symbols) and correct rejoining with the hybridization assay (open symbols). The profoundly different kinetics for total and correct rejoining in C.B-17 and RAD54−/− cells are indicative of DSB misrejoining. For scid cells, total and correct rejoining can be described by a single curve indicating that nearly all rejoining events lead to restriction fragment reconstitution without any detectable sign of DSB misrejoining. For C.B-17 and RAD54−/− cells, the cumulative SE of the measurements for total and correct rejoining, B, are fitted: the cumulative SE of the measurements for total and correct rejoining, C, are fitted by hand. Bars indicate the difference between total and correct rejoining. Bars, the cumulative SE of the measurements for total and correct rejoining, C, are fitted by hand. B, fraction of the initially induced DSBs, which are misrejoined after various times after irradiation. The data are obtained from A from the difference between total and correct rejoining. **Bars**, the cumulative SE of the measurements for total and correct rejoining.
nent, whereas cells with a defect in any of the genes involved in NHEJ rejoin DSBs mainly with a slow component. Although the concept of a two-component description of the rejoining kinetics may seem somewhat arbitrary, it has been successfully applied by other authors as well (49). Because of the similarity of the slow rejoining components in mutant and wild-type cells, it may be tempting to speculate that the slow component of DSB rejoining in wild-type cells is independent of NHEJ and that mainly rejoining by the fast component is affected by a deficiency in NHEJ. A comparison of the kinetics for total rejoining with the kinetics of correct DSB rejoining, however, shows that the slow component in wild-type cells is not accompanied by restriction fragment reconstitution, suggesting that the process involves rearrangement rejoining. In contrast, the slow component of DSB rejoining in NHEJ-deficient cells is closely followed by restriction fragment reconstitution, suggesting that it represents a different repair pathway. Because the fast rejoining component in wild-type cells is also strongly diminished in the NHEJ-defective cells, our results suggest that both fast and slow DSB rejoining in repair-proficient cells are mediated by NHEJ. Our results show additionally that the alternative repair process proceeding with slow kinetics in NHEJ-deficient cells is a distinct process that is suppressed in the presence of functional NHEJ, possibly because this process has a higher potential for DSB misrejoining than NHEJ in situations when only a few breaks occur simultaneously (Fig. 2, F and G). The mechanism and enzymatic nature of the alternative repair pathway are presently unknown. It has been suggested by others that an inherently slow NHEJ pathway operates in DNA-PK-deficient cells that is stimulated by DNA-PKcs to rapidly remove DSBs (49). Because our experiments yield similar rejoining kinetics for Ku80-, DNA-PKcs-, and DNA ligase IV-deficient cells, such a putative NHEJ apparatus may neither involve DNA-PK nor DNA ligase IV. It is also worth stating that a single-strand annealing process generating deletions of several kbp was observed to proceed in G1-arrested yeast for several hours (50) and may thus represent an alternative repair mechanism to NHEJ. DSB repair studies using cell-free extracts from Ku80-deficient rodent cells (51) or from *Xenopus laevis* eggs (52) also suggest the existence of a microhomology-driven, single-strand annealing pathway operating in the absence of DNA-PK-dependent NHEJ. Because loss of DNA sequences up to several tens of kbp would lead to restriction fragment reconstitution that cannot be distinguished from the intact fragment in our hybridization assay, no DSB misrejoining events would be scored in that case. On the other hand, however, single-strand annealing also has the potential for rearrangement rejoining because two DSBs on different yeast chromosomes can frequently form reciprocal translocations by an annealing mechanism between homologous regions on two different chromosomes (53).

Our results show that HR is not involved in repairing DSBs during G1 because we have demonstrated wild-type characteristics for rejoining of both correct and incorrect break ends in *RAD54*−/− mouse fibroblasts. However, other evidence suggests that the contribution of HR to DSB repair in mammalian cells should not be underestimated: (a) DSBs enzymatically generated in chromosomal constructs containing tandemly repeated sequences can be efficiently repaired by HR (54); and (b) asynchronous *RAD54*−/− mouse embryonic stem cells (55, 56), HR-deficient hamster 

\*\textit{CHO} \(2\beta\) mutants (20), and *RAD54*−/− (but not *RAD52*−/−) chicken cells (57, 58) are radiation sensitive. It may be, however, that HR is restricted to the late S-G2 phase of the cell cycle when the presence of a sister chromatid facilitates this process. This is also suggested by a remarkable elevation of radiation resistance in XRC4-deficient CHO (59), Ku70−/− chicken (60), and scid mouse cells (61) during late S-G2 and by the loss of S-G2 radiosensitivity in IRS-1 cells (62) deficient in HR. If indeed HR operates predominantly in G2, a preference for correct DSB rejoining may be expected in this phase of the cell cycle. However, it is tempting to speculate that in situations when no sister chromatid is available, DSB repair by HR would operate between non-allelic repeat sequences, in which case even a single break would be sufficient to cause genomic rearrangements. In such a scenario, the cell may be better off not to rely on HR but to use NHEJ, which is erroneous only when multiple DSBs occur in close proximity.

**ACKNOWLEDGMENTS**

We thank J. Kiefert (Justus Liebig University, Giessen, Germany) for providing laboratory space and the γ-ray source at the Strahlenzentrum in Giessen. We also thank C. Kirchgeissner (Stanford University School of Medicine) for providing C.B-17 and scid cells and R. Kanaar (Erasmus University, Rotterdam, the Netherlands) for providing *RAD54*−/− cells. The mouse probe Fre2 was generously given to us by R. W. Friedrich (Justus Liebig University, Giessen, Germany), and CHO-K1 cells were a gift from R. Greinert (Dermatological Center, Buxtehude, Germany). Dihydrofolate reductase cDNA (American Type Culture Collection), MRC-5, and xrs-6 cells (European Collection of Cell Cultures) are commercially available.

**REFERENCES**


GENOMIC REARRANGEMENTS FORMED BY DNA END-JOINING


Radiation-induced Genomic Rearrangements Formed by Nonhomologous End-Joining of DNA Double-Strand Breaks

Kai Rothkamm, Martin Kühne, Penny A. Jeggo, et al.

*Cancer Res* 2001;61:3886-3893.

**Updated version**
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/61/10/3886

**Cited articles**
This article cites 61 articles, 28 of which you can access for free at:
http://cancerres.aacrjournals.org/content/61/10/3886.full.html#ref-list-1

**Citing articles**
This article has been cited by 36 HighWire-hosted articles. Access the articles at:
/content/61/10/3886.full.html#related-urls

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.