Efficient Rejoining of Radiation-induced DNA Double-strand Breaks in Centromeric DNA of Human Cells*

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Although major efforts in elucidating different DNA double-strand break (DSB) repair pathways and their contribution to accurate repair or misrepair have been made, little is known about the influence of chromatin structure on the fidelity of DSB repair. Here, the repair of ionizing radiation-induced DSBs was investigated in heterochromatic centromeric regions of human cells in comparison with other genomic locations. A hybridization assay was applied that allows the quantification of correct DSB rejoining events in specific genomic regions by measuring reconstitution of large restriction fragments. We show for two primary fibroblast lines (MRC-5 and 180BR) and an epithelial tumor cell line that restriction fragment reconstitution is considerably more efficient in the centromere than in average genomic locations. Importantly, however, DNA ligase IV-deficient 180BR cells show, compared with repair-proficient MRC-5 cells, impaired restriction fragment reconstitution both in average DNA and in the centromere. Thus, the efficient repair of DSBs in centromeric DNA is dependent on functional non-homologous end joining. It is proposed that the condensed chromatin state in the centromere limits the mobility of break ends and leads to enhanced restriction fragment reconstitution by increasing the probability for rejoining correct break ends.

DNA double-strand breaks (DSBs) are biologically important lesions that can arise endogenously during replication or meiosis or from reactive oxygen species and can also be produced by exogenous agents such as ionizing radiation. They can either be repaired accurately, restoring genomic integrity, or can be misrepaired, leading to genomic rearrangements. Higher eukaryotic cells primarily repair DSBs by one of two distinct pathways, non-homologous end-joining (NHEJ) and homologous recombination (HR). NHEJ is the predominant repair pathway in the G1 phase of the cell cycle and involves the DNA end-binding heterodimer Ku70/Ku80, the catalytic subunit of the DNA-dependent protein kinase (DNA-PK), the XRCC4 gene product, and DNA ligase IV (for reviews, see Refs. 1–3). The precise mechanism of DSB rejoining by NHEJ is still unclear, but the process does not require significant regions of sequence homology. NHEJ is thought to frequently restore the original sequence at the break point, but sometimes small deletions or insertions may arise (4). In the presence of multiple DSBs, this pathway has the potential to generate genomic rearrangements by joining incorrect break ends (5, 6). HR achieves repair by the use of an undamaged homologous template. It involves proteins of the RAD52 epistasis group and plays a crucial role in DSB repair in higher eukaryotic cells (for a review, see Ref. 7). In contrast to mammalian cells defective in NHEJ, however, HR-deficient mutants display less severe radiosensitivity (8). Although DSBs enzymatically generated in chromosomal constructs containing tandemly repeated sequences can be efficiently repaired by HR (9), the prevailing evidence suggests that this process is predominantly involved in the resolution of stalled replication forks and operates for the repair of exogenously induced damage almost exclusively in late S and G2 when the sister chromatid is available as a homologous template (10). The fidelity of DSB repair by HR should be high, because the original sequence can be restored efficiently and because potential genomic rearrangements resulting from crossing-over events during gene conversion between homologous sequences on heterologous chromosomes could not be detected (11).

The repair of various DNA lesions is known to depend on the chromatin organization of human cells, but little is known about the influence on DSB repair processes. Constitutively heterochromatic regions can be found in several genomic positions, sometimes covering large portions of a chromosome as in the human Yq arm and sometimes encompassing rather small regions as in telomeres. Also, centromeric regions are constitutively heterochromatic and remain condensed during the interphase of the cell cycle (12). Within a heterochromatic region, usually a specific type of repetitive DNA predominates, with α-satellite DNA being exclusively localized to the centromeric regions of all human chromosomes. α-Satellite DNA ranges in size between 200 bp and several Mb and is comprised of tandemly arranged 171-bp monomers that are organized into higher order repeats of anything between 2 and ~35 monomers (13). The sequence divergence between individual higher order repeats is very small (<2%), but the monomers within a higher order repeat can show substantial variation (20–40% sequence divergence). Therefore, it is the higher order repeat unit that characterizes distinct subfamilies of α-satellite DNA. Any one chromosome often contains several different subfamilies and a single subfamily can also be shared by different chromosomes (14). Since its discovery in humans (15), α-satellite DNA has become the most extensively studied of the...
highly repetitive DNA families and provides a model for the large number of repeat families that characterize the genome of human cells.

In the present study, we have investigated the influence of the particular structural characteristics in the cenomeres of human cells on DSB repair processes by employing a method that is based on hybridization detection of individual genomic restriction fragments. We had previously shown that the repair of DSBs induced by ionizing radiation can be measured in specific genomic regions (6, 16) but had so far failed to detect significant variations in DSB repair between different locations (17). Here, we report, for the first time, the existence of intragenomic heterogeneity of DSB repair. It was observed that human cells repair DSBs in centromeric DNA substantially better than in DNA regions without particular structural characteristics. By investigating cell lines deficient in defined DSB repair pathways and by examining ribosomal DNA (rDNA) regions, the efficient repair in centromeric DNA could be attributed to the condensed chromatin state of this genomic region. Our observations help to elucidate how the repair of DSBs and the generation of chromosomal aberrations by incorrect repair processes are affected by chromatin organization.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Viability Assays—Primary human fibroblasts MRC-5 (obtained from the European Collection of Cell Cultures) and 180BR (a gift from P. Jeggo, Medical Research Council Cell Mutation Unit, University of Sussex, Brighton, UK), and human bladder carcinomata MGH-U1 (a gift from S. Powell, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA) were grown in minimal essential medium supplemented with 10% (20% for 180BR) fetal calf serum and antibiotics. All incubations were performed at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. Non-dividing cultures, obtained by growing cells to superconfluency, were used in all experiments (at least 90% in G1, as determined by flow cytometry). For cell viability tests by DNA content measurements using a FACScan flow cytometer (Becton Dickinson), cells were harvested, resuspended in PBS (137 mm NaCl, 2.7 mm KCl, 8 mm Na2HPO4, and 1.5 mm KH2PO4, pH 7.45), fixed with 70% ice-cold ethanol, and stained with propidium iodide. To assess cell viability by trypan blue exclusion, an equal volume of trypan blue dye (0.5% (w/v) in PBS) was added to an aliquot of cells, and the solution was allowed to sit at room temperature for dye uptake. The fraction of dead cells with dye uptake was then scored in a hemacytometer. For the MTT viability test, ~3 × 105 cells in a 75-cm2 culture flask were incubated for 3 h with 5 mm of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma) in 10 ml medium at 37 °C to allow the cells to utilize the yellow tetrazolium salt. After removing the dye formazan, 10 ml of 0.1 N NaOH solution (10% SDS, 0.01% HCl) was added, and the flask was incubated overnight at 37 °C to produce a homogeneous solution suitable for measurement of optical density. The difference in light absorption at 550 and 700 nm is proportional to the product concentration and was determined by chemical dosimetry. Cells were irradiated in culture flasks filled with ice-cold PBS to prevent repair during irradiation. For deter-

Conventional PFGE Assay for Total DSB Rejoining—To determine all DSB repairing events in the entire genome, DNA was purified by PFGE without prior restriction enzyme digestion. After washing the plugs briefly in TE buffer (10 mm Tris-HCl, 1 mm Na2EDTA, pH 8), PFGE was carried out for 40 h in 0.8% agarose in 0.5× TBE (44.5 mm Tris-HCl, 44.5 mm boric acid, 1 mm Na2EDTA, pH 8) at 16 °C with a field strength of 3 V/cm and pulse times linearly increasing from 40 to 80 s. The endonuclease (Bgl II) was added to the PFGE buffer (Bio-Rad). Gels were stained with ethidium bromide, photographed with a digital camera system under UV transillumination, and analyzed with commercially available software (AIDA 2.40, Raytest). The fraction of DNA able to enter the gel was quantified. Samples that were irradiated with various doses and not incubated for repair served as a calibration curve to determine the percentage of remaining DSBs from the fraction of DNA entering the gel in the repair samples (17).

Hybridization Assay for Region-specific DSB Rejoining—For determining DSB rejoining in specific genomic regions, DNA was digested with restriction enzymes prior to electrophoresis. 50-μl plug stripes containing DNA from about 5 × 108 cells were washed at least six times in several milliliters of TE for 1 h each, equilibrated in reaction buffer with 100 units of restriction enzyme for 12–15 h at 37 °C. NotI, a rare-cutting enzyme, was used to investigate DSB rejoining in average DNA regions and HindIII, a frequent-cutting enzyme, was used to analyze DSB rejoining in centromeric DNA. For investigating rDNA regions, the frequent-cutting enzyme EcoRV was used. The gels were run for 115 h at 1.5 V/cm with pulse times from 500 to 3000 s to separate NotI fragments of MRC-5 and 180BR cells, or for 40 h at 3 V/cm with 40–800 s to separate NotI and EcoRV fragments of MGH-U1 cells and HindIII fragments of MRC-5 and 180BR cells. The HindIII-digested DNA of MGH-U1 cells was separated for 20 h at 4.5 V/cm with a constant pulse time of 180 s. After PFGE separation, the DNA was partly depurinated and vacuum-blotted onto a charged nylon membrane (Hybond N+, Amersham Biosciences, Inc.). The membranes were baked for at least 4 h and hybridization overnight were performed in 20 ml of hybridization buffer (5 × SSPE, 5 × Denhardt’s, 2% SDS, and 0.1 mg/ml sonicated fish sperm DNA) at 65 °C for the membranes containing NotI-digested DNA and at 54 °C in 20 ml of hybridization buffer containing 50% formamide for the membranes containing HindIII- or EcoRV-digested DNA. DNA probes D21S1 (pPW229C) (15), D21S4 (pPW229D) (15), D21Z1 (pPW229A) (19), and D21S11 (pPW229B) (19) were digested with EcoRI or HindIII digestion buffers and used for hybridization. Prehybridization for 1800 s in a CHEF-DR system (Bio-Rad). Gels were stained with ethidium bromide, photographed with a digital camera system under UV transillumination, and analyzed with commercially available software (AIDA 2.40, Raytest). The fraction of DNA able to enter the gel was quantified. Samples that were irradiated with various doses and not incubated for repair served as a calibration curve to determine the percentage of remaining DSBs from the fraction of DNA entering the gel in the repair samples (17).

RESULTS

Strategy for Measuring DSBs in the Centromere and in Average DNA—We had previously described that DSB induction and repair can be investigated by analyzing specific genomic restriction fragments in irradiated and repair-incubated mammalian cells (6, 16). The approach is based on separating genomic restriction fragments by PFGE and detection of specific fragments by Southern hybridization. In the present study we applied this hybridization approach to investigate DSB induction and rejoining in centromeric DNA in comparison to average DNA regions of human cells in the G1 phase of the cell cycle. For analyzing average DNA regions, the rare-cutting
restriction enzyme NotI was used and an individual fragment from chromosome 21 with a size of either 2 or 3.2 Mbp was visualized by Southern hybridization with the single copy DNA probe D21S1, which detects a single 3.2-Mbp fragment (for MRC-5 and 180BR cells) or with probe D21S4 detecting a 2-Mbp restriction fragment (for MGH-U1 cells). HindIII-digested DNA was hybridized with probe D21Z1, resulting in a multiband image in which each band represents a centromeric restriction fragment from a different chromosome. The bands indicated by the arrows on the right were used for quantitative analysis. Fragment sizes were determined with yeast chromosomal standards. DSBs inside the restriction fragments decrease the intensities of the hybridizing bands, that can be used to obtain numbers of breaks per fragment (see text). Graphs show the number of induced DSBs normalized to the same length of DNA (DSBs/Mbp) as a function of radiation dose. The induction rates (given below in $10^{-3}$ DSBs/Mbp/Gy) were not significantly different for the cell lines and genomic regions analyzed. MRC-5 cells: 8.7 (D21S1), 8.2 (D21S4), and 9.9 (D21Z1; both fragments were analyzed and the average is shown), 180BR cells: 8.5 (D21S1) and 8.1 (D21Z1), MGH-U1 cells: 8 (D21S4) and 7.7 (D21Z1). Error bars represent the standard error of the mean (S.E.) from four to six independent experiments (except MRC-5 probed with D21S1, two experiments).
with nearly the same size but different hybridization intensities were evaluated (Fig. 1B, lower left panel). The band showing strong hybridization intensity probably represents a centromere with a large proportion of the L1.26 subfamily detected with DNA probe D21Z1, whereas the weakly hybridizing band containing radiation dose, and a smear below the band accumulates as the target size for DSB induction. The application of a frequent cutting restriction enzyme to analyze centromeric DNA has the dual advantage that (i) average DNA regions are electrophoretically separable from centromere DNA so that the specificity of the hybridization signal can easily be controlled and (ii) the analyzed restriction fragment contains exclusively centromeric sequences and is not comprised of a mixture of centromeric and average DNA, which would be the case if rare cutting enzymes were applied.

After exposure of human cells to ionizing radiation, the band intensities of the restriction fragments decrease with increasing radiation dose, and a smear below the band accumulates that reflects smaller DNA pieces due to induced DSBs (see parts B and C in Figs. 3–5 below). Analysis of the number of breaks within the restriction fragment examined involved the measurement of the hybridization signal of the band, \( I_{B} \), representing the unbroken restriction fragment and the quantification of the hybridization signal of the whole lane, \( I_{T} \), to normalize to equal DNA loading. Assuming a random induction of breaks within the fragment (Poisson distribution), the number of DSBs is equivalent to the negative logarithm of the ratio of the relative intensity, \( I_{R} = I_{B}/I_{T} \), of the irradiated sample to the relative intensity of the control sample. After normalizing the number of DSBs per restriction fragment to equal DNA length (DSBs per Mbp), results from different restriction fragments can be compared. We investigated DSB induction in a centromeric DNA region in comparison to average DNA in three different cell lines and found that the number of breaks induced per Mbp per Gy was the same (Fig. 1C). This shows that centromeric DNA and average DNA are equally susceptible to DSB induction after x-irradiation and suggests that there are no large differences in induction of DSBs in different genomic locations.

**Cell Viability after High Doses of Ionizing Radiation**—A necessity for studying DSB repair following ionizing irradiation is the cells’ functional integrity. The capacity of a cell to grow into a colony is a proof of reproductive integrity, and because radiation doses of a few Gy significantly reduce the colony forming ability of a cell, doses of several tens of Gy certainly will kill cells in the sense that they are rendered unable to divide. For differentiated or non-proliferating cells, however, loss of the capacity for sustained proliferation is an inappropriate term. In this case, cell death in the mode of apoptosis or necrosis can be recognized based on morphological, biochemical, and molecular changes of the dying cell (23). Because confluent non-dividing cell cultures were used in the present work for repair studies and radiation damage is known to be a potent inducer of apoptosis in numerous cell types, we wished to assess the impact of ionizing irradiation on cell viability by a series of different assays. Normal primary human fibroblasts were irradiated with 80 Gy of x-rays and analyzed in comparison with unirradiated cells either 1 or 6 days after irradiation. No morphological change was apparent in the cell culture (Fig. 2A). Because cell death is usually accompanied by a change in the property of cells to scatter light (24), we determined cell viability by flow cytometric measurements of light scatter. Again, no difference between un-irradiated and irradiated cells was detectable (Fig. 2B). Extensive DNA degradation is a characteristic event of cell death, but no change in DNA integrity was observed electrophoretically (data not shown) or with flow cy-
Efficient DSB Rejoining in Centromeric DNA of Human Fibroblasts—Repair of DSBs in a specific genomic region can be investigated by measuring the reconstitution of the corresponding restriction fragment to its original size. After repair incubation of irradiated cells, the band of the original restriction fragment is partly reconstituted, so that its intensity corresponds to the band intensity of a sample irradiated with a lower dose and not incubated for repair (Fig. 3, B and C). Because DSBs are induced linearly with dose (see Fig. 1C), the percentage of DSBs that had not been rejoined to reconstitute the original restriction fragment can easily be determined as a function of incubation time. We applied this approach to primary human fibroblasts irradiated with 80 Gy of x-rays and observed appreciably more rejoining events that lead to restriction fragment reconstitution in centromeric DNA than in two average DNA regions (Fig. 3D). Results from a conventional PFGE assay in which all DSB rejoining events in the whole genome are measured by analyzing the ethidium bromide-stained gel (Fig. 3A) show nearly complete DSB repair after several hours of incubation. This demonstrates that the cells have the capacity to efficiently rejoin DSBs even after high radiation doses, as has also been observed by others (e.g. Ref. 26). It is, however, important to note that the criterion for DSB rejoining with the conventional PFGE assay is increase in average molecular weight of broken DNA pieces, whereas the hybridization approach only detects those DSB rejoining events that lead to restriction fragment reconstitution. A comparison of the time course for total DSB rejoining as measured by conventional PFGE and correct DSB rejoining leading to restriction fragment reconstitution suggests that in average DNA regions about 50% of all rejoining events do not reconstitute the original band and hence represent misrepair events that generate restriction fragments smaller or larger than the original band. Because restriction fragment reconstitution is considerably enhanced in centromeric DNA regions (about 75% of all induced DSBs are correctly rejoined), our results show that
misrepair events that generate genomic rearrangements occur much less frequently in centromere DNA. The capacity of the cells to preferentially reconstitute restriction fragments in centromeric DNA regions was also observed after lower radiation doses, such that after a dose of 40 Gy almost 90% of all rejoining events in the centromere lead to reconstituted fragments, in contrast to 60% correct DSB rejoining in average DNA (Fig. 3E). We conclude that primary human fibroblasts rejoin DSBs induced by ionizing radiation in centromeric DNA in a way that more frequently reconstitutes the original restriction fragment than DSB rejoining in average DNA regions.

**DNA Ligase IV-deficient Human Fibroblasts Show Improved DSB Rejoining in Centromeric DNA**—We next asked whether the efficient DSB rejoining observed in centromeric DNA of repair-proficient human fibroblasts can also be detected in cells that are grossly defective in rejoining the bulk of radiation-induced DSBs. The radiosensitive primary human fibroblast cell line, 180BR (27), which carries a defect in DNA ligase IV (28), was investigated for its ability to rejoin DSBs in centromeric DNA in comparison with average DNA regions. Conventional PFGE analysis revealed a pronounced DSB repair defect in these cells with a large fraction of unrejoined breaks after incubation times up to 24 h (Fig. 4, A and D). Examination of the time course for restriction fragment reconstitution after 80 Gy showed significantly improved band reconstitution in centromeric DNA compared with average DNA (Fig. 4, B–D). Notably, the capacity of these cells to reconstitute a centromeric restriction fragment even exceeds their ability for total DSB rejoining determined by conventional PFGE (Fig. 4D). Because total DSB rejoining involves restriction fragment reconstitution as well as misrepair events that lead to fragments smaller or larger than the original band, this result further substantiates our suggestion that DSB rejoining in centromeric DNA is more efficient than in average DNA regions. The improved reconstitution of a centromeric restriction fragment compared with a restriction fragment of an average DNA region was also observed after radiation doses of 40 and 60 Gy (Fig. 4E). We conclude that the increased efficiency for rejoining DSBs in centromeric DNA regions can also be observed in DNA ligase IV-deficient human fibroblasts.

**Efficient DSB Rejoining in Centromeric DNA Is Unaffected by Wortmannin Treatment**—Because both a repair-proficient and a repair-deficient human cell line showed improved DSB rejoining in centromeric DNA regions, we wished to further examine the influence of a repair defect on the preferential rejoining of centromeric DSBs by chemically suppressing the cells’ ability for DSB repair. This has the advantage that the identical restriction fragment in the same cells can be analyzed under conditions that allow or inhibit DSB rejoining. Among several cell lines tested, the human tumor cells MGH-U1 gave optimal hybridization signals for centromeric DNA, such that the largest restriction fragment was electrophoretically well separated and showed the strongest signal with hardly any detectable background hybridization (Figs. 1B and 5C). These cells were treated with wortmannin, a potent inhibitor of the family of phosphatidylinositol 3-kinases, including DNA-PK, which is, like DNA ligase IV, an essential component of functional NHEJ. An estimation of the level of the repair defect caused by wortmannin treatment of MGH-U1 cells was determined by conventional PFGE and showed that DSB rejoining is substantially inhibited in the presence of wortmannin (Fig. 5, A and D). Investigation of the time course for restriction fragment reconstitution after 80-Gy x-irradiation showed considerably enhanced DSB rejoining in centromeric DNA compared with average DNA, both for untreated cells (Fig. 5E) and for cells treated with wortmannin (Fig. 5F). The kinetics for untreated MGH-U1 cells are strikingly similar to the results obtained with repair-proficient primary human fibroblasts (compare Fig. 5E with Fig. 3D), suggesting that the capacity for efficient rejoining of centromeric DSBs is universal for human cells. Additionally, the kinetics observed for MGH-U1 cells treated with wortmannin resemble those of DNA ligase IV.
deficient 180BR fibroblasts, and both cell lines show improved restriction fragment reconstitution in centromeric DNA, which proceeds with a time course that is similar in shape to the time course for reconstituting a restriction fragment of average DNA (Figs. 5 and 4D). This suggests that the NHEJ-independent rejoining activity of wortmannin-treated MGH-U1 cells and DNA ligase IV-deficient 180BR cells is more efficient in centromeric DNA than in average DNA regions. It is interesting to note that the impact of wortmannin on total rejoining is apparently much larger than it is on restriction fragment reconstitution. Hence, mainly DSB misrepair is reduced by wortmannin treatment, consistent with our recently published observation that NHEJ mediates this class of rejoining events (6).

**DSB Rejoining in Ribosomal DNA Is Less Efficient Than in Centromeric DNA**—There are basically two different features that distinguish centromeric from average DNA regions and that could account for the efficient rejoining of DSBs in the centromere. First, centromeric DNA is comprised of a large number of homologous sequences, whereas average DNA regions contain repetitive elements much less frequently. Thus, the majority of DSBs in average genomic regions will be induced in DNA sequences for which no homologous partner, except the homologous chromosome, is available. Any homology-dependent repair mechanism may therefore benefit from the availability of sequence homologies in the centromeres. Second, centromere regions are part of the constitutive heterochromatin, and the centromeric DNA is densely packed. Therefore, any DSB repair mechanism that relies on tethering break ends together may operate more efficiently in genomic regions in which the diffusion of DNA ends is restricted. To evaluate whether the presence of sequence homologies or the heterochromatic state is mainly responsible for efficient DSB rejoining in centromeric DNA regions, we have extended our investigations to ribosomal DNA (rDNA). The rDNA region is also comprised of homologous sequences, although the repeat length of 44 kbp (29) is considerably longer than that of centromeric DNA. In contrast to the centromere, however, rDNA is not densely packed but euchromatic and likely in a transcriptionally active state.

Investigation of the reconstitution of an rDNA-specific restriction fragment was performed in MGH-U1 cells after 80-Gy x-irradiation by the hybridization approach. Similar to the strategy for measuring DSBs in centromeric DNA, a frequent-cutting restriction enzyme was used for DNA digestion. EcoRV generates fragments with sizes of several kbp for average DNA (data not shown) but leaves the rDNA region uncut (30), resulting in much larger rDNA restriction fragments that can be detected with DNA probe 18SrRNA (Fig. 6A). We reasoned that DSB rejoining in the rDNA region should proceed with kinetics similar to those observed in centromeric DNA if the presence of sequence homologies were important for efficient DSB rejoining. If, however, efficient DSB rejoining were mainly effected by a densely organized chromatin state, the results from the
DSB Rejoining in Centromeric DNA

In this study, we have exploited the capacity of a specialized DSB rejoining assay to investigate the genomic heterogeneity of DSB repair in G1-phase human cells. Hybridization detection of specific genomic restriction fragments allows the investigation of radiation-induced DSBs in defined genomic locations and their repair by the criterion of restriction fragment reconstitution. We show that DSB repair in centromeric DNA regions is substantially improved compared with DSB repair in average genomic locations.

Mammalian cells show two categories of DSB rejoining: the rejoining of correct break ends leading to restriction fragment reconstitution and the joining of ends from different DSBs, which generates fragments smaller or larger than the original fragment (6, 16). If only a few DSBs are present in any one cell, restriction fragment reconstitution clearly dominates. After exposure with a high dose of x-rays, however, the presence of multiple breaks at the same time enhances the probability for joining incorrect break ends (31, 32). Under these conditions, the level of restriction fragment reconstitution is considerably different from the level of total rejoining, which involves rejoining events of both categories. This situation, in which a restriction fragment from an average DNA region is only partly reconstituted, was chosen to investigate genomic heterogeneity of DSB repair. Previous work in our group had observed nearly identical kinetics for restriction fragment reconstitution in several genomic regions and various organisms. Two NotI fragments from the same genomic region on chromosome 21 of primary human fibroblasts, a NotI fragment from the X chromosome of primary human fibroblasts containing the hypoxanthine guanine phosphoribosyl transferase gene, the same regions in human tumor cells, an MluI fragment of Chinese hamster ovary cells containing the dihydrofolate reductase gene, and an SpI fragment of mouse embryonic fibroblasts all showed the same time course for restriction fragment reconstitution with ~50% correct rejoining after exposure with 80-Gy x-rays (6, 17, 33). Additionally, investigations from another group also indicate similar kinetics for restriction fragment reconstitution in various genomic regions of primary human fibroblasts and Chinese hamster ovary cells (34). The result of the present work that the reconstitution of a centromeric restriction fragment is substantially better than that of average DNA fragments was also obtained for several cell lines and clearly demonstrates that some particular character of the centromeric DNA region decreases the probability for joining incorrect break ends.

Two different characteristics of human centromeres could conceptually account for the efficient DSB rejoining observed in this region. First, centromeric DNA is comprised of many repetitive elements so that the majority of DSBs are induced in DNA sequences for which an abundant number of nearby homologous sequences is available as a template for a homology-dependent repair mechanism. Second, centromere regions are highly heterochromatic, located in association with nucleoli or fused in chromocenters (35), and the centromeric DNA is probably less dynamic than DNA from an average genomic region. Although ionizing irradiation could influence the dynamic nature of the chromatin, the probability for rejoining correct break ends may be enhanced by the heterochromatic character, leading to improved restriction fragment reconstitution in the centromere. In this case, any repair mechanism that tethers break ends together without the requirement for a homologous template may more frequently rejoin correct break ends. We have addressed this question by investigating DSB rejoining in cell lines with a specific defect in DNA-PK-dependent NHEJ,
the predominant repair pathway of mammalian cells in the G1 phase of the cell cycle.

Both DNA ligase IV-deficient primary human fibroblasts and a human tumor cell line treated with wortmannin reconstitute a centromeric restriction fragment more efficiently than a restriction fragment from an average DNA region. Wortmannin is an inhibitor of phosphatidylinositol 3-kinases, including DNA-PK, the protein mutated in ataxia telangiectasia (Atm) and the Atm-related protein, but exhibits its effect on cell radiosensitivity and DSB rejoining mainly by the inhibition of DNA-PK (36). This is consistent with the observation of the present work that the effect of wortmannin on DSB rejoining in centromeric and average DNA regions is similar to the effect of a deficiency in DNA ligase IV. A comparison of the time course for reconstitution of a centromeric restriction fragment in DNA ligase IV-deficient fibroblasts and DNA-PK-inhibited tumor cells with the time course for reconstitution of a centromeric restriction fragment in NHEJ-proficient primary human fibroblasts and human tumor cells not treated with wortmannin shows impaired DSB rejoining in the centromere region of the NHEJ-deficient cell lines (compare Fig. 3D with Fig. 4D and see Fig. 5F). Because the operation of a homology-dependent repair mechanism is not expected to be compromised by a defect in DNA ligase IV or by inhibition of DNA-PK, this result suggests that the efficient reconstitution of centromeric restriction fragments in repair-proficient cells depends on functional NHEJ, which may more frequently rejoin correct break ends if the DNA is less dynamic. This conclusion is further supported by our studies investigating restriction fragment reconstitution in the rDNA region of human cells, which is also comprised of repetitive elements. In contrast to centromeric DNA, however, it is not heterochromatic. In this genomic region, the time course for restriction fragment reconstitution is similar to the time course for reconstituting a restriction fragment from an average DNA region and substantially less efficient than in centromeric DNA (Fig. 6B). Additionally, even in the absence of functional NHEJ, the presence of sequence homologies in rDNA does not enhance DSB rejoining (Fig. 6C). This is in line with the notion that homology-dependent repair pathways are restricted to the G1 phase of the cell cycle when the sister chromatid is available (10, 37, 38).

Taken together, these results support a model in which heterochromatic centromeres contain DNA that is less dynamic, resulting in a decreased probability of an NHEJ-dependent repair mechanism for joining incorrect break ends. This model is consistent with a recent study of yeast interphase nuclei reporting that the chromatin is generally highly mobile, particularly in the G1 phase, with the exception of specialized chromosomal domains, such as the centromeres and telomeres, which show constrained chromatin movement throughout the cell cycle (39). Also investigations in living human cells using time-lapse confocal microscopy suggest that centromeres are primarily stationary during interphase (40, 41). An alternative view of how the heterochromatin character of centromeric DNA could enhance the rejoining of correct break ends is provided by a recent demonstration that the 30-nm chromatin fiber is more regular and more condensed for centromeric DNA than for bulk DNA (42). Therefore, DSBs in the centromere may less frequently disrupt the chromatin fiber, leading to fewer chromatin breaks that can give rise to incorrect DSB rejoining.

Although the existence of genomic heterogeneity of DSB repair was uncovered in the present study after high radiation doses, there is evidence that the observed phenomenon also applies to situations when only a few breaks are present in a cell nucleus. A high dose situation may be criticized based on the concern that the activity of DSB repair mechanisms is compromised in the presence of a large number of DSBs. However, identical rejoining kinetics have been observed for doses up to 340 Gy (16), demonstrating that the cellular repair system is not saturated in this dose range. Moreover, mutants defective in NHEJ, which is the predominant DSB repair pathway in the G1 phase of the cell cycle and is shown in the present work to preferentially reconstitute centromeric restriction fragments, display extreme radiosensitivity and chromosomal repair defects at very low doses and show a substantial DSB repair deficiency at high doses, arguing that the same mechanism is utilized both at high and low doses. Finally, the potential of the NHEJ mechanism to rejoin breaks incorrectly has been demonstrated for a situation when two DSBs coincide in the cell nucleus (5) and was also observed after high radiation doses (6). Therefore, it is likely that the efficient reconstitution of a centromeric restriction fragment would also be observed after much lower radiation doses, although no formal proof of this notion is provided in the present work.

Notwithstanding the predominance of DNA-PK-dependent NHEJ for mammalian DSB repair in the G1 phase of the cell cycle, cells deficient in this pathway show substantial DSB rejoining (see Figs. 4D and 5D). Because 180BR cells are not completely deficient in DNA ligase IV but show residual activity supporting V(D)J recombination (28), the observed DSB rejoining activity in 180BR cells after ionizing irradiation (see also Refs. 43, 44) may also be attributable to residual DNA ligase IV activity. However, a similar level of DSB rejoining has been observed in various mammalian cell lines deficient in NHEJ (6, 45), including fibroblasts derived from a Ku80 knockout mouse model (46), demonstrating that DSB rejoining in NHEJ-deficient cells cannot be attributed to residual activities of the enzymes involved in DNA-PK-dependent NHEJ. The nature of the DSB rejoining activity in NHEJ mutants has been a matter of speculation. Although the slow time course for DSB rejoining observed in Ku80-, DNA-PKcs-, and DNA ligase IV-deficient cells is compatible with slow repair kinetics by HR observed in organisms using such a mechanism for repairing radiation-induced breaks (47), there are several lines of evidence that argue against this (48). The most compelling work investigated DSB rejoining in the hyper-recombinogenic DT40 chicken cell line for which several mutants defective in HR are available and demonstrates that a cell line defective in both NHEJ and HR still exhibits substantial DSB rejoining (49). Also results from cytogenetic studies that demonstrate an increased level of radiation-induced chromosomal exchanges in cells defective in DNA-PK-dependent NHEJ (50–53) argue against a role of HR in the absence of NHEJ, because the error-free HR pathway would be expected to limit and not enhance chromosomal exchanges. Finally, studies performed in our group have demonstrated that DNA ligase IV-deficient cells show substantial DSB misrepair when low dose rate experiments are performed (6) or when repair is allowed for several days.2 Taken together, it is reasonable to postulate that some form of error-prone repair process operates in the absence of one of the components of DNA-PK-dependent NHEJ. The results of the present work, that both DNA ligase IV-deficient primary human fibroblasts and a human tumor cell line treated with wortmannin show improved reconstitution of a centromeric restriction fragment compared with a restriction fragment from an average DNA region, therefore suggest that this error-prone repair process benefits from the heterochromatic character of the centromere in a similar way as does the DNA-PK-dependent NHEJ pathway in repair-proficient cell lines. In this context it is worth stating that a single-strand

\(^2\) M. Löbrich et al., unpublished data.
Annealing process was observed to proceed in G1-arrested yeast cells for several hours (54) and may thus represent an alternative repair pathway to DNA-PK-dependent NHEJ in human cells. Furthermore, recent experiments with Brca2-deficient mouse cells suggest that single-strand annealing-mediated processes can lead to chromosomal exchanges (55). It is possible that the rejoining capacity of such a repair process benefits both from the heterochromatic character of the centromere and from the presence of relatively short repetitive elements in centromeric DNA but not from the 4-kb repeats in the rDNA regions.

Our result of improved restriction fragment reconstitution in centromeric DNA directly impacts on the intragenomic heterogeneity of visible chromosome aberrations. DSB rejoining events that lead to restriction fragment reconstitution include precise rejoining in which the original sequence is restored as well as imprecise rejoining with gain or loss of short DNA sequences at the break point leading to a fragment that cannot be distinguished from the original fragment. The category of rejoining events that generates fragments smaller or larger than the original fragment by joining ends from different DSBs, in contrast, includes visible chromosomal aberrations, such as e.g. translocations or inversions. The data of the present experiment, therefore, predict that chromosomal exchange aberrations occur less frequently in the centromere and probably also less frequently in genomic regions that are highly heterochromatic. The idea that chromatin structure plays an important role in aberration formation has acquired the minds of radiation cytogeneticists ever since the early days of radiation biology (56). Many conflicting observations have been made (e.g. Refs. 57, 58), but there is some evidence that chromosomal exchange aberrations may less frequently be formed in condensed genomic regions. For example, the long arm of the X chromosome, which is entirely occupied by heterochromatin in Chinese hamster cells, was reported to exhibit a significant deficiency of exchange breakpoints (59), and highly condensed X-chromosomal Barr-bodies in cell lines with multinucleum X chromosomes were observed to be insensitive with regard to aberration formation by ionizing radiation (60). Finally, an inefficiency of x-rays to form chromosomal aberrations in a constitutively heterochromatic region close to the centromere of human chromosome 1 in comparison to damage in the whole genome was observed (61). The investigations of the present work provide, on the level of DSB rejoining, an explanation for these cytogenetic observations.

Taken together, our findings demonstrate an increased efficiency for DSB rejoining in centromeric DNA of human cells. Under the experimental conditions applied, DSB repair in average genomic regions frequently involves the joining of incorrect break ends. The probability for this type of DSB misrepair event is substantially decreased in the centromere. Investigations with cell lines defective in NHEJ and studies analyzing rDNA regions further indicate that efficient DSB repair in the centromere results from the heterochromatic character, which probably limits the mobility of centromeric DNA and thereby increases the likelihood for rejoining correct break ends. These observations underscore the important role of chromatin organization for efficient DSB rejoining and the prevention of chromosomal aberrations.

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