Repair of x-ray-induced DNA double-strand breaks in specific Not I restriction fragments in human fibroblasts: Joining of correct and incorrect ends

MARKUS LÖBRICH, BJÖRN RYDBERG, AND PRISCILLA K. COOPER

Life Sciences Division, Lawrence Berkeley National Laboratory, University of California, Berkeley, CA 94720

Communicated by Philip C. Hanawalt, Stanford University, Stanford, CA, August 7, 1995

ABSTRACT An assay that allows measurement of absolute induction frequencies for DNA double-strand breaks (dsbs) in defined regions of the genome and that quantitates rejoining of correct DNA ends has been used to study repair of dsbs in normal human fibroblasts after x-irradiation. The approach involves hybridization of single-copy DNA probes to Not I restriction fragments separated according to size by pulsed-field gel electrophoresis. Induction of dsbs is quantitated from the decrease in the intensity of the hybridizing restriction fragment and an accumulation of a smear below the band. Rejoining of dsbs results in reconstitution of the intact restriction fragment only if correct DNA ends are joined. By comparing results from this technique with results from a conventional electrophoresis assay that detects all rejoining events, it is possible to quantitate the misrejoining frequency. Three Not I fragments on the long arm of chromosome 21 were investigated with regard to dsb induction, yielding an identical induction rate of 5.8 × 10⁻³ break per megabase pair per Gy. Correct dsb rejoining was measured for two of these Not I fragments after initial doses of 80 and 160 Gy. The misrejoining frequency was about 25% for both fragments and was independent of dose. This result appears to be representative for the whole genome as shown by analysis of the entire Not I fragment distribution. The correct rejoining events primarily occurred within the first 2 h, while the misrejoining kinetics included a much slower component, with about half of the events occurring between 2 and 24 h. These misrejoining kinetics are similar to those previously reported for production of exchange aberrations in interphase chromosomes.

DNA double-strand breaks (dsbs) constitute the most important primary damage produced by ionizing radiation (1), as evidenced by the fact that mammalian cell mutants that are defective in rejoining dsbs are highly radiosensitive (2-5). However, although it seems intuitively apparent that DNA dsbs must be the precursors of interphase chromosome breaks and ultimately of chromosomal aberrations, the precise nature of the relationship among these is not known. There is, for example, an obvious discrepancy between the initial yield of dsbs and of interphase chromosome breaks as measured by the technique of premature chromosome condensation (PCC). While x-irradiation produces ~40 dsbs per Gy per human cell (6, 7), the corresponding number for PCC breaks varies between 3 and 6 (8, 9). Despite the discrepancy in initial yields, however, there are strong correlations between measured radiation-induced dsbs and PCC breaks. The rejoining kinetics for PCC breaks and DNA dsbs are similar (10), and in addition radiosensitive mutants with defects in dsb rejoining show deficiencies in rejoining PCC breaks (11, 12). Such results strongly suggest that DNA dsbs are indeed the primary initial lesions underlying PCC breaks. Furthermore, experiments showing that the level of residual PCC damage after repair corresponds to the number of acentric fragments suggest that PCC breaks themselves represent precursor lesions to chromosome aberrations (13, 14). This conclusion is strengthened by the observation made by using PCC in combination with fluorescence in situ hybridization that exchange events observable in interphase chromosomes accumulate with kinetics similar to the rejoining of PCC breaks (15).

Cell reproductive death from ionizing radiation may be attributable largely to production of chromosomal aberrations that result in large-scale loss of genetic information as acentric fragments (13). Moreover, chromosomal rearrangements such as translocations, inversions, and large deletions most likely are the principal genetic alterations resulting in radiation-induced malignant transformation (16). It is therefore important to understand the sequence of events leading from the primary DNA damage to these critical chromosomal lesions. To do so it is necessary to investigate both the mechanisms of repair of dsbs and the extent and fidelity of the rejoining process(es).

Considerable progress in elucidating molecular mechanisms involved in dsb rejoining has been made recently through the identification of the defects in three complementation groups of ionizing-radiation-sensitive rodent cell mutants, designated x-ray repair cross-complementing (XRCC) groups 5, 6, and 7 (17). These mutants are defective in dsb rejoining and have been shown to involve defects in components of DNA-dependent protein kinase, an abundant nuclear protein in human cells (5, 18-20, 22-24). They also exhibit defects in the rejoining steps in V(D)J recombination (where V is variable region, D is diversity region, and J is joining region) (5, 25), a site-specific recombination process required for rearrangement of DNA to generate variability in genes encoding immunoglobulin and T-cell receptors, as do XR-1 mutants in XRCC4, which similarly have a defect in dsb rejoining. These circumstances suggest that common elements are involved in the rejoining of dsbs in both processes and directly implicate DNA-dependent protein kinase in both, presumably fulfilling a similar role in each case.

In contrast to the situation in bacteria and yeast in which homologous recombination plays the major role, the predominant process for joining of double-stranded DNA ends in higher eukaryotic cells does not appear to involve regions of significant homology. Analyses of plasmid integration sites and of translocation and deletion breakpoint junctions, for example, have frequently implicated short overlaps of one to six complementary bases in the end-joining events, suggesting the operation of illegitimate recombination processes (refs. 26 and 27 and references therein). This conclusion is particularly significant in view of the requirement for DNA-dependent

Abbreviations: dsb, double-strand break; PCC, premature chromosome condensation; FAR, fraction of radioactivity released (from the plug in a pulsed field gel); PFGE, pulsed-field gel electrophoresis; Mbp, megabase pair(s).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
protein kinase activity both in rejoining of radiation-induced dsbs and in V(D)J recombination, the only known example of site-specific, or illegitimate, recombination in mammalian cells. However, both XR-1 and xrs (XRCC5) mutants, which have defects in both processes, exhibit increased resistance to cell killing when irradiated in late S or early G2 phase, and XR-1 in particular has been shown to have increased dsb rejoining in that portion of the cell cycle (for review, see ref. 2). It is therefore possible that a homologous recombination mechanism operating on sister chromatids does also function in rejoining dsbs after irradiation in mammalian cells. The fidelity of such disparate mechanisms in the rejoining of breaks might well be quite different.

In this paper we present and employ an approach that not only provides a direct determination of dsb induction in defined locations within the mammalian genome but also allows evaluation of the proportion of dsbs that is incorrectly rejoined. We present evidence that in normal human fibroblasts irradiated in the G0 phase of the cell cycle ~25% of all x-ray-induced dsbs after doses of 80 or 160 Gy are misrejoined.

MATERIALS AND METHODS

Cell Culture. Primary human dermal fibroblasts GM38 from a normal individual were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository, Coriell Institute for Medical Research (Camden, NJ). Cells were grown in a humidified 5% CO2/95% air atmosphere at 37°C in McCoy's 5A medium supplemented with 10% (vol/vol) fetal bovine serum (GIBCO), 2 mM glutamine, 10 mM Heps, and antibiotic/antimycotic solution (penicillin at 100 units/ml, streptomycin sulfate at 100 mg/ml, amphotericin B at 0.25 μg/ml) (GIBCO). Nondividing confluent cells were used in all experiments. For experiments with prelabeled cells, [14C]thymidine (Amersham; 2.15 GBq/ mmol) at 3700 Bq/ml was added to the medium 5-7 days ahead of time and removed 2-3 days prior to the experiment.

X-Irradiation and Repair Incubation. X-irradiation was performed at 225 kV and 15 mA with 0.35-mm Cu filtration at a dose rate of 1-2 Gy/min. For measurements of induction of dsbs, cells were irradiated in agarose plugs. The plugs were kept in phosphate-buffered saline (PBS) at 0°C before, during, and after irradiation. For repair experiments, cells were irradiated as confluent monolayers in cell culture flasks (with the exception of one repair experiment in which the cells were embedded in plugs before irradiation). The medium was replaced with PBS at 0°C and irradiations were done on ice. The PBS was then replaced with prewarmed fresh medium and the flasks (or plugs) were incubated at 37°C for the time of repair. Trypsinization was performed for 5 min at room temperature followed by chilling on ice.

Fraction of DNA Radioactivity Released (FAR) Assay. The FAR assay was performed essentially as described (28). Trypsinized [14C]thymidine-labeled cells were embedded in agarose, lysed, and digested with proteinase K. The DNA was separated by pulsed-field gel electrophoresis (PFGE) with a CHEF-DR II system (Bio-Rad). The fraction of radioactivity released from the plug was measured by liquid scintillation counting of the sliced gel. Results of experiments measuring FAR as a function of dose were used as a calibration curve to obtain relative numbers of remaining breaks from FAR values after repair.

Hybridization Assay. A detailed description of the hybridization assay can be found elsewhere (29). Trypsinized cells were embedded in agarose, lysed, and digested. The DNA was digested with Not I, separated by PFGE, transferred to a membrane, and hybridized with one of three DNA probes, D21S1, D21S4, or D21S15, that hybridizes to single-copy sequences on Not I restriction fragments of chromosome 21 with sizes of 3.2 megabase pairs (Mbp), 2 Mbp, and 1.2 Mbp, respectively (30, 31). Probe labeling using random priming (Amersham Multiprime DNA labeling system) was performed with [α-32P]dCTP (>222 Tbp/mmol, Amersham) according to the manufacturer's instructions. Quantitative analysis of the hybridization signal was performed by using a phosphorimaging system (Molecular Dynamics). For the hybridization experiment using 32P-labeled cells, the total DNA distribution given by the 14C radioactivity could be quantified with the PhosphorImager before hybridizing the membrane. The 32P hybridization signal in this experiment was quantified with the PhosphorImager by using a 2 oz. copper foil (Molecular Dynamics; 1 oz. = 28.4 g) to block out the electrons originating from 14C decays.

RESULTS

Induction of dsbs in Specific Not I Fragments. The hybridization method for studying the induction and repair of dsbs in human cells involves restriction endonuclease digestion of DNA from irradiated cells followed by PFGE, Southern blot analysis, and hybridization with DNA probes recognizing single-copy sequences. DNA, isolated from confluent normal human fibroblasts, irradiated or not with x-rays, and embedded in agarose plugs, was treated with Not I and the resulting fragments were separated according to size by PFGE. With increasing radiation dose, the DNA size distribution shifts to lower molecular weight. The molecular size of radiation-induced dsbs (Fig. 1 Upper, lanes 1-6).

After electrophoresis the DNA was transferred to a membrane and hybridized with the probe D21S1, which recognizes a 3.2-Mbp Not I restriction fragment originating from the long arm of chromosome 21 (Fig. 1 Lower). With increasing dose, the band for the intact 3.2-Mbp fragment decreases in intensity, while increasing amounts of smaller DNA pieces appear as a smear below the main band (Fig. 1, lanes 2-6). The incubated samples (lanes 7-10) show a decrease of the smear and a reappearance of the original band. Analysis of the number of breaks within the restriction fragment containing the hybridization site involved the measurement of the hybridization signal intensities of the bands and of the whole lanes by using a PhosphorImager. By assuming a random distribution of breaks, the number of dsbs within the restriction fragment...
examined was determined by the negative logarithm of the ratio of the band representing the unbroken fragments to the total hybridization signal of the lane, which represents all fragments containing the hybridization site. Analysis of results from this and other similar experiments (Fig. 2) showed that the number of dsbs induced per Mb per Gy was the same for three Not I fragments studied: a 3.2-Mbp fragment located at 21q21.1 containing the hybridization site for D21S1, a 2-Mbp fragment at 21q21 recognized by D21S4, and a 1.2-Mbp fragment at 21q22.3 carrying the sequence for D21S15 (32–34). The induction of dsbs was found to be linear with dose with a yield of $5.8 \times 10^{-3}$ dsb per Mb per Gy. This value corresponds to $\sim 36$ dsbs per cell per Gy and is in agreement with previous determinations (6, 7).

Rejoining of dsbs. Two methods were used to follow rejoining of the radiation-induced dsbs, a conventional analysis (FAR assay; ref. 28) that measures total rejoining and the hybridization assay as outlined above, in which the criterion for rejoining is the restitution of the original restriction fragment. Since random rejoining of DNA ends would be unlikely to reconstitute a hybridizing restriction fragment of the same size, this approach quantitates rejoining of correct DNA ends within the limits of resolution of the gel system. Fig. 3 is an ethidium bromide-stained gel from a typical experiment using the FAR assay to follow rejoining of dsbs after 80 Gy for up to 20 h. An induction curve for FAR vs. dose was used as a calibration curve for dsbs remaining after repair incubation. The rejoining of dsbs as a function of incubation time after irradiation as measured by the two methods is shown in Fig. 4. For either method the rate and extent of rejoining measured were independent of whether the cells were incubated during repair as a monolayer or as a suspension in agarose plugs. As measured by the FAR assay, rejoining in the genome overall was essentially complete by 20 h after 80 Gy, with <5% of the initial breaks remaining. In contrast, the 3.2-Mbp Not I restriction fragment recognized by the probe D21S1 was only 70% reconstituted. Thus, these results indicate that 25% of dsbs are misrejoined if it is assumed that repair in this region is representative of the genome overall. The broken pieces of the 3.2-Mbp Not I fragment that were not reconstituted were present as a smear under the band. Therefore, an alternative possibility could be that the result indicates atypically poor rejoining in this particular region of the genome. However, since the 3.2-Mbp Not I fragment is much larger than the average Not I fragment, misrejoining would be expected to produce new Not I fragments smaller than the original one that would, therefore, be difficult to distinguish from nonrejoined pieces.

To test the generality of the extent of misrejoining determined from the results of Fig. 4, similar hybridization experiments were performed for a second restriction fragment from the long arm of chromosome 21 and for a different x-ray dose. Because cells are necessarily radioactively labeled for the FAR assay but usually are not for the hybridization approach, we also tested whether labeling had any effect on the extent of restitution of a hybridizing band. As summarized in Table 1, the result was the same under all these circumstances. Thus, about 30% of the initial breaks did not rejoin to reconstitute either of two restriction fragments after the same dose (probes D21S1 or D21S4; 160 Gy) or a given restriction fragment after two different doses (80 or 160 Gy; probe D21S1). Furthermore, there was no difference whether the cells were labeled

![Fig. 3](image_url)  
**Fig. 3.** Rejoining of dsbs induced by x-rays as measured with the FAR assay. Lanes: 1 and 10, ethidium bromide-stained gel that contains Schizosaccharomyces pombe and Saccharomyces cerevisiae marker chromosomes; 2–9, DNA from cells irradiated with 80 Gy and incubated for 0, 0.5, 1, 2, 4, 6, 8, and 20 h, respectively; 11–14, DNA from sham-irradiated cells incubated for 0, 2, 8, and 20 h, respectively.

![Fig. 4](image_url)  
**Fig. 4.** Rejoining of dsbs after x-irradiation at 80 Gy, measured either within the 3.2-Mbp Not I fragment recognized by D21S1 (for the hybridization method, solid symbols) or within the whole genome (for the FAR approach, open symbols). For either assay, repair incubation was performed with cells embedded in agarose plugs (circles) and with cells as confluent monolayers in culture flasks (triangles).
with $^{14}$C or not. In parallel experiments, the effect of dose on total rejoining was determined by using the FAR assay. Rejoining by this measure was essentially complete independent of the number of initially induced dsbs, such that after 20 h at least 95% of breaks were rejoined for doses from 50 to 340 Gy (Fig. 5). A comparison of this value with the percentage of breaks not correctly rejoined after long incubation times as measured by the hybridization assay (see Table 1 and Fig. 4) indicates that $\approx 25\%$ of all initially induced breaks undergo misrejoining. Analysis of the time course of correct rejoining as given by restriction fragment reconstitution indicates that this rejoining component takes place mainly within the first 2 h and does not proceed further at times longer than 4 h (Fig. 4 and Table 1). In contrast, total rejoining has both fast and slow components, with the latter occurring over many hours (Figs. 4 and 5).

To determine whether the misrejoining frequency of 25% is valid throughout the whole genome, a different approach based on analysis of the total Not I fragment size distribution was applied. DNA from $^{14}$C-labeled cells was digested with Not I and separated by PFGE. Fig. 6 shows size distributions of restriction fragments obtained by applying the electrophoresis conditions used in Fig. 1. Distribution A of Fig. 6 represents DNA from unirradiated cells, distribution B is DNA from cells exposed to 160-Gy x-rays, and distribution C is DNA from cells irradiated with 160 Gy and incubated for 24 h. The compression zones on the left side of the distributions A and C contain fragments that are too big to be separated by size. Mathematical considerations (analysis not presented) show that the original Not I restriction fragment distribution in unirradiated cells is nonrandom, with more large fragments than would be expected for randomly distributed restriction sites. The distribution representing the DNA of irradiated cells (distribution B) is shifted to lower molecular weight due to the radiation-induced breaks. This distribution is independent of the original Not I distribution in the high molecular weight region and is approximately random. After an incubation time of 24 h essentially all of the dsbs have been rejoined (see Fig. 5), shifting the DNA distribution back toward a higher molecular weight (distribution C). However, this Not I distribution differs significantly from the Not I distribution of unirradiated cells, thus directly suggesting that not all of the original Not I fragments have been reconstituted. A mathematical analysis (data not given) shows that the difference in the high molecular weight region cannot be explained by a nonrejoining frequency of 5% (or less) but is consistent with a misrejoining frequency of $\approx 25\%$ throughout the whole genome.

**DISCUSSION**

In this study we have applied hybridization detection of unique large restriction fragments resolved by PFGE to quantitate the induction and rejoining of DNA dsbs after x-irradiation of normal human fibroblasts. One significant advantage of this approach is that an absolute yield of dsbs can be determined for defined small regions of the genome, in contrast to other approaches that give only an average value for the genome overall. The yield of dsbs was found to be indistinguishable ($5.8 \times 10^{-3}$ break per Mbp per Gy) for three Not I fragments from chromosome 21, suggesting that there are no large differences in induction of dsbs in different genomic locations after x-irradiation. It is still possible that either highly condensed heterochromatic sequences or highly transcriptionally active regions might have quite different dsb yields, but in this connection it is worth noting that one of the probes used in this study, D21S15, is from a region of chromosome 21 in which the gene density appears to be locally very high (35).

A second major advantage of the hybridization approach is that it provides a measure of rejoining of correct DNA ends as defined by reconstitution of the original restriction fragment. When used in combination with a measure of total rejoining, it can thus provide an estimate of the proportion of breaks that misrejoin. By using this approach, we found that in normal human fibroblasts irradiated in the G0 phase of the cell cycle $\approx 25\%$ of dsbs induced by 80 or 160 Gy of x-rays are rejoined by the connection of incorrect DNA ends. This value is actually an underrepresentation of the full extent of misrejoining, since alterations up to 100 kbp would not alter the size of the restriction fragments (several megabase pairs long) sufficiently for detection by PFGE and hence are counted as correctly rejoined. The misrejoining frequency was measured in two Not I fragments with no detectable difference. An analysis of the overall Not I distribution supports the rejoining results obtained by the hybridization assay, indicating that the two Not I fragments studied represent regions that are repaired in a manner representative of the genome overall.

Examination of the time course for total rejoining vs. correct rejoining reveals that the latter occurs primarily within the first 2 h while the former includes a second much slower component that continues for many hours. Since the slow component of total rejoining is not detected with the hybridization method, it is likely to represent misrejoining events almost exclusively. After 2 h of incubation, the difference between the fraction of breaks that have been correctly rejoined and the total fraction rejoined is 10–15%, in comparison to a final difference of 25%. Hence $\approx 50\%$ of all misrejoining events occur in the first 2 h. This halftime for misrejoining is comparable to slow rejoining kinetics for ionizing-radiation-induced interphase chromosome.

---

**Fig. 5.** Rejoining of dsbs as a function of time after various x-ray doses (50–340 Gy) as measured with the FAR assay.

**Fig. 6.** Normalized intensity distributions of Not I restriction fragments of DNA separated according to size by PFGE under conditions identical to those of Fig. 1. Distributions were obtained by measuring the $^{14}$C signal from labeled DNA with a PhosphorImager. Distributions: A, Not I restriction fragments of unirradiated cells; B, from cells irradiated with x-rays at 160 Gy; C, from cells allowed to repair for 24 h after x-rays at 160 Gy.
breaks (PCC) in normal human fibroblasts (36) and CHO cells (37). The kinetics of production of exchange aberrations in interphase chromosomes of irradiated human cells are in remarkable agreement with the kinetics of dsb misrejoining determined here, with ~50% of exchanges occurring between 2 h and 48 h of incubation (15). It is tempting to speculate that the slowly rejoining component of PCC breaks is representative of the set of slowly rejoining dsbs and that these events are responsible for most chromosome aberrations. Numerical considerations reveal that only a small fraction of misrejoining events as detected in this study could lead to visible metaphase aberrations, while the majority must be presumed to produce changes in the DNA (e.g., deletions or inversions) that are not cytologically observable. Since the resolution of the assay used here is much higher, on the order of 100 kbp, this difference is perhaps not surprising. However, it must also be considered in this connection that the experiments for quantitating dsb misrejoining frequencies in this study were necessarily performed at much higher doses than experiments scoring chromosomal aberrations, which are typically 10 Gy or less. The possibility that fidelity of dsb rejoining varies with dose remains, although no dose effect was detected between 80 and 100 Gy in this study, and measurements of mutation frequency have suggested a linear dose–response relationship even to very low doses (21). It should be particularly interesting to apply the hybridization approach to determine whether the dsb misrejoining frequency varies through the cell cycle, in particular whether it is decreased in late S or early G2 phase when homologous recombination mechanisms might prevail, and also to investigate whether rejoining fidelity is affected by the state of chromatin condensation or transcriptional activity in particular genomic regions. It may also be possible to gain information concerning the mechanisms involved in correct repair of dsbs vs. misrejoining by applying this approach to studies of radiosensitive mutant cells.

We thank Ely E. Kwoh for expert technical assistance and for preparing the figures. This work was supported by Order W-18,265 from the NASA Specialized Center of Research and Training in Radiation Health at Lawrence Berkeley Laboratory and by the Office of Energy Research, Office of Health and Environmental Research, Health Effects Research Division, U.S. Department of Energy, under Contract DE-AC03-76SF00098.
