Radiation induced DNA double strand breaks are rejoined by ligation and recombination processes

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ABSTRACT
Using the method of filter elution of double stranded DNA under neutral conditions we have shown that most of gamma-ray induced double strand breaks (DSB) are rejoined in both mammalian and bacterial cells. Rejoining also occurs in the G1 phase in V79 Chinese hamster cells and under different growth conditions. Within 8 minutes at 37°C, half the breaks are rejoined. The rejoining in E. coli is equally fast and depends on the presence of DNA ligase. Some of the breaks in E. coli rejoin slowly, and these require rec+. The non-rejoined DSB are distributed over the DNA without any preference for the nucleosomal or the linker structure in the chromosome. Two kinds of DSB rejoining are discriminated, a fast process of DNA ligation and a slower process involving rec functions.

INTRODUCTION
DNA DSB are the result of irradiation. With increasing linear energy transfer of the radiation, cell killing and number of unrepairable DSB also increase (1, 2). DSB thus seem to be life threatening damages.

This report addresses the occurrence and mechanism of DSB repair in bacterial and mammalian cells. We have used the technique of "neutral elution" for the detection of DSB (3). The technique is based on the observation that double stranded DNA passes through a membrane filter under nondenaturing conditions at a rate depending on its size. Using this technique we observed rejoining of DNA DSB in both mammalian and bacterial cells, and have detected two independent mechanisms resulting in fast and slow rejoining processes. In bacteria, these can be distinguished by mutations. The slow process requires rec+, the fast process DNA ligase.
METHODS

V79 Chinese hamster lung cells were grown as monolayers in Petri dishes in Earle's medium supplemented with 10 % (v/v) fetal calf serum and penicillin. Cultures were growing exponentially as determined by flow cytometry. DNA was uniformly labelled with (2-14C) thymidine (0.02 μCi/ml) for 24 h. Before irradiation cells were incubated in fresh medium for 2 h.

G1 phase cells were collected as follows. Tissue culture flasks with 14C thymidine labelled exponentially growing V79 cells were first carefully washed with warm conditioned medium to discard dead cells. Mitotic cells were shaken off and collected in ice cold medium. Using this procedure more than 95 % of the collected cells were mitotic. Cells were then incubated for 2 h in warm conditioned medium to step synchronously in the G1 phase. About 80 % of the cells were in G1 phase during irradiation, as determined by flow cytometry.

Cells were cultivated in monolayers until stationary phase, further growth being inhibited by their density.

Irradiation was carried out in a 60Co gamma source (Gamma-cell 220, Atomic Energy of Canada Ltd.) at a dose rate of 33 Gy/min. During irradiation the cell monolayers were kept at ice temperature in growth medium. After irradiation the medium was replaced by conditioned medium of 37 C and the cells incubated for different periods of time.

Cells were then treated with 0.25 % cold trypsin solution and resuspended in ice cold PBS. 5 x 10^5 cells were applied by funnel to a polycarbonate filter (Bio-Rad Laboratories, 25 mm diameter, pore size of 2 μm). The cells were attached by carefully sucking the PBS through the filter and washing and sucking it twice more with 5 ml of ice cold PBS. After this, the lysis and DNA elution procedure of Bradley and Kohn (1979) was carried out at pH 9.6 (3).

After fractionated elution the radioactivity of the different samples including the filter was counted in 10 ml Instagel.

For bacterial cell experiments the following strains were used: the wild type strain E.coli K12 AB1157 (F-, proI-, his-, arg-, leu-, threo-, uvr+), AB2463 the recA 13 mutant derived from AB1157 (Howard Flanders and Theriot, 1966), and KS 268
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carrying lig ts7 mutation isolated by E.C. Pauling and obtained from Dr. Geider, Heidelberg. Suspension cultures were grown overnight in TY medium at 37 C, the ts7 strain was grown at 30 C. Bacteria were resuspended at a density of OD600 = .1 and incubated for 3 h with (2-14C) thymidine (0.02 μCi/ml) for DNA labelling. After a chase of 10 min in normal TY medium, the cell suspension was cooled in ice and gamma-irradiated. 100 μl of the irradiated suspension was resuspended in 1 ml warm TY medium for repair incubation. Filter elution was carried out as for V79 cells using the same lysis procedure except that filters with a pore size of 0.45 μm were used.

RESULTS

Rejoining of gamma-ray induced DSB in V79 cells. The neutral filter elution technique allows measurement of relative DNA size by means of their different elution rates. In fig. 1a the different elution profiles of irradiated V79 monolayers are shown for different doses. Percentage DNA retained on the filter is plotted versus elution time (fraction number). With higher doses the DNA was eluted faster and in increasing amounts. From such elution profiles we compared DNA sizes. In order to do this we kept the experimental conditions constant, and always evaluated the data by the same normalizing procedure. For evaluation we used the mean value (MV) of the percentage DNA retained on the filter of the ten eluted fractions because the data obtained in this way was the most reproducible. These mean values, derived from the elution profiles of fig. 1a, are plotted versus dose in fig. 1b. The linearity of the dose effect curve for DSB production speaks for the accuracy and relative sensitivity of the filter elution method in comparison to sedimentation techniques (4).

If cells are incubated at 37 C after the irradiation, they clearly rejoin the DSB (fig. 2). The profiles rise with increasing incubation time, reaching a constant slope at 120 min. The slope of the unirradiated control, however, was never reached even after very long incubation times. Half of the rejoining occurred within 7.5 min (fig. 3). Some 20 % of DSB are not rejoined and the kinetics reach asymptotic levels at
Figure 1. Induction of DSB in DNA of V79 cells by gamma-rays. (a) The percentage of DNA retained on the filter is plotted as a function of the eluted fraction. Each fraction represents material eluted during 90 min at a rate of 4 ml/h.
(b) Dose effect curve of DSB induction. The mean values (MV) of the percentages of ten fractions of the elution profile normalized to control are plotted as a function of dose. Variability is indicated by the size of the symbols.

120 min. Human fibroblasts have the same fast rejoining kinetics (data not shown). Monolayers and suspension cultures rejoin with equal speed. These kinetics are not influenced by the type of radiation used to generate the breaks (X- or gamma-rays).

DSB rejoining is independent of the cell cycle. We investigated DSB rejoining in different phases of the cell cycle, comparing exponentially growing cells with G1 phase cells and stationary cells. Fig. 4 shows that cells which are not growing, as well as cells which are in G1, or growing exponentially, all have the same fast rejoining kinetics. This independence from the cell cycle suggests that the rejoining cannot depend on the presence of a duplicated DNA molecule.

Nonrejoined DSB are distributed over the DNA. It has been proposed that the repair of DSB depended on the site within the
Figure 2. Rejoining of DSB in V79 DNA after a given dose of 100 Gy gamma-rays for different incubation times.

![Graph showing rejoining of DSB in V79 DNA after 100 Gy gamma-rays for different incubation times.]

Figure 3. Rejoining kinetics of DSB in V79 DNA after a dose of 100 Gy. The percentage of rejoined DNA was calculated as follows:

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\% \text{ rejoined DNA} = \log \left( \frac{MV(\text{irr., rejoined})}{MV(\text{irr., not rejoined})} \right) / \log \left( \frac{MV(\text{control})}{MV(\text{irr., not rejoined})} \right)
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chromosome structure and that repair was different in linker or nucleosome DNA (5). Radiation induced DSB are distributed in the DNA at random. We tested whether a constant fraction at each dose was nonrejoined and used this as a measure of a possible nucleosome influence on repair. The majority of DNA is packed into nucleosome at a constant ratio of about 40 bp linker to 140 bp nucleosome DNA. If linker DNA alone were not rejoined, a constant fraction of 22% nonrejoined DSB should be found for each dose. The result contradicts this assumption. The proportion of nonrejoined DNA varied with dose (fig. 5) suggesting that rejoining occurs in linker as well as in nucleosomal DNA.

Rejoining of gamma-ray induced DSB in bacteria. To get more information about the mechanisms of DSB rejoining we investigated bacterial DNA. We adapted the neutral filter elution technique for the smaller bacterial DNA by using filters with a pore size of 0.45 μm. The elution profiles of irradiated and rejoined E.coli DNA were similar to those of mammalian cell DNA. Because of the smaller size of bacterial DNA, the variability of the elution kinetics was somewhat greater than with mammalian DNA, and the slope was less steep by a factor which corresponded to the difference in genome (target) size (fig. 6). The speed of rejoining was also fast, with a half-life of about 8 min (fig. 7).
Figure 5. Nonrejoined DNA in V79 cells plotted as a function of dose. The percentage of nonrejoined DNA without incubation is set to 100% for each dose.

Figure 6. Dose effect curve of DSB induction in E.coli K12 strain AB 1157 (wt). The values are calculated as in Fig. 1b.
Rejoining occurred at same rate in a recombination deficient (recA) mutant. The extent of maximal rejoining, however, seemed to differ between the mutant and wild-type strains. Our working hypothesis is that recA may be involved in a slow rejoining process.

E. coli mutants also indicate possible mechanisms by which the fast DSB rejoining process takes place. We determined the extent of rejoining in a mutant thermostensitive in DNA ligase. At the permissive temperature, (30 C) rejoining of DSB was nearly normal. At 40 C it was strongly suppressed (fig. 8). DSB rejoining in wt E. coli as in the recA mutant was similar at both temperatures (not shown).

Figure 7 Rejoining kinetics of DSB in bacterial DNA after 4000 Gy. The values are calculated as in fig. 3. The symbols are: □ AB 1157 (wt), ○ AB 2463 (recA).
Figure 8. Rejoining kinetics of DSB in ligase deficient E. coli mutant KS268 at permissive and nonpermissive temperature after 4000 Gy. The values are calculated as in fig. 3.

DISCUSSION
The filter elution technique carried out under neutral conditions (3) gives a measure of the size of large DNA fragments. There are various advantages of this method over sedimentation techniques (4, 6). In this report, we use the technique for the determination of DSB. Because of the sensitivity of the method, the irradiation dose can be low enough to measure real DSB, with little contamination by closely placed single-strand breaks.

A computer analysis of the curve shape of the elution profiles revealed that the elution rate was not constant for either irradiated or even unirradiated control DNA, whose size distribution should be random. This means that DNA elution
probability is determined not only by the pore size of the filter, but is also influenced by an interaction between the filter material and DNA depending on the pH value of the elution buffer.

The method is more sensitive at higher pH values up to pH 9.6. At 9.6 the DNA was not denatured. Under constant experimental conditions we can observe relative changes in DNA size with high reproducibility. The mean value of the percentage of DNA retained on a filter after 15 hours elution is our measure of the relative size distribution of the DNA. The linear dose relation of radiation induced DSB demonstrates that the lower dose limit for radiation induced DSB measurement in eucaryotic DNA is about 5 Gy (fig. 1b). Our data also show that the neutral filter elution can be adapted to measurements on the smaller DNA fragments obtained from bacteria.

Many authors investigating DSB rejoining kinetics using sedimentation analysis or the unwinding technique have found that these breaks are rejoined at slow rates (4-7 h) (7,8). It seems that these methods reveal predominantly a rec-dependent pathway (9) while our technique measures both this and a fast ligation process. The fast rejoining measured by neutral elution is not affected by parameters such as cell type or growth conditions. The same rejoining velocity in cells of different cell cycle length indicates that the rejoining mechanism measured by filter elution must be independent of the cell cycle. Experiments using synchronized cells confirmed this interpretation (fig. 4). In addition to the experiments with mutants in E. coli, the fact that fast rejoining is independent of the cell cycle renders unlikely the explanation that the mechanism for this is recombination. A recombination dependent process would change with ploidy, genome number or growth conditions (9,10,11). Resnick and Moore (12) found a lack of recombination in G0 CHO cells but rejoining of DSB suggesting a ligation process.

Using bacterial DNA we have shown that the fast rejoining of DSB as measured by filter elution is not due to recombination but to ligation. This fast DSB rejoining is not inhibited in the recA strain incubated in TY medium, indicating that it is not due to recombination repair. Using sedimentation techniques
however, a rec-dependent slow rejoining process has been found (9). This is supported by our demonstration that rec~ mutants of E. coli lost the ability to promote the slow rejoining process.

The most conclusive evidence for ligation as a fast rejoining mechanism results from observations on the temperature sensitive mutant. This strain only rejoins DSB at the permissive temperature (30 C), whereas other strains used also rejoin DSB at non-permissive temperatures. Thus, DNA-ligase appears to be involved in the rejoining of radiation-induced DSB in procaryotic cells. The cell cycle independent fast kinetics found in V79 hamster cells suggest that the mechanism may be similar in mammalian cells. We favor the conclusion that gamma irradiation causes 3 types of DSB: i) breaks which are repaired by DNA-ligase, ii) breaks which require recombination, possibly in combination with DNA-ligase, iii) non-repaired breaks. Since rec~ mutants of E. coli are 4 fold more radiosensitive than wild-type (13) but show very similar fast rejoining kinetics, the rec pathway seems to be more important for survival than ligation alone. We suggest the following explanation for the failure of other methods to detect the fast rejoining process: The ligase-rejoined DNA may appear intact in the neutral elution method, but subject to dissociation or rebreakage by the other methods. The neutral elution technique does not unambiguously prove reconstitution to covalent integrity, although it seems likely. We plan to examine DSB rejoining with respect to integrity and to error-free repair.

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