The repair of double-strand breaks and S1 nuclease-sensitive sites can be monitored chromosome-specifically in *Saccharomyces cerevisiae* using pulsed-field gel electrophoresis

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Summary

Repair under non-growth conditions of DNA double-stranded breaks (DSBs) and S1 nuclease-sensitive sites (SSSs; e.g. DNA damage which is processed by *in vitro* treatment with S1 nuclease to DSBs) induced by [^{60}Co]-gamma-rays (200 Gy; anoxic conditions) was monitored in a diploid repair-competent strain of *Saccharomyces cerevisiae.* We used pulsed-field gel electrophoresis (PFGE), which allows the separation of chromosome-sized yeast DNA molecules, to determine the number of DSBs and SSSs in individual chromosome species of yeast. Our results indicate that SSSs which have been regarded as clusters of base damage in opposite DNA strands are repaired efficiently in a repair-proficient diploid strain of yeast. The time course of SSS repair is comparable to the one of DSB repair, indicating similarities in the molecular mechanism. Both types of repair kinetics are different for different chromosome species.

Introduction

Regions hypersensitive to DNA endonucleases are dispersed non-randomly in eukaryotic chromatin (Goding and Russell, 1983; Pulleybank *et al.*, 1985). Presumably these regions are characterized by local alterations of the helical B-DNA conformation existing either constitutively or temporarily in the chromatin of eukaryotes (cf. Gross and Garrard, 1988). These native sites of nuclease hypersensitivity seem to play a role in the regulation of chromatin structure and function, in particular in the regulation of gene expression (Elgin, 1982; Gross and Garrard, 1988).

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In addition, S1 nuclease-sensitive sites (SSSs) are induced by gamma-irradiation of prokaryotic and eukaryotic cells (Andrews *et al.*, 1984; Martin-Bertram *et al.*, 1984; Paterson *et al.*, 1976; Yoshizawa *et al.*, 1976; Furuno *et al.*, 1979; Geigl and Eckardt-Schupp, 1990). These induced sites are defined by their *in vitro* susceptibility to S1 nuclease, yielding DNA double-stranded breaks (DSBs). Their molecular structure is not known; any possible similarity or local identity to S1 hypersensitive sites in native chromatin has not been proven so far. It has been proposed that gamma-induced SSSs correspond to clustered base damage (Martin-Bertram *et al.*, 1983). Base alterations in opposite DNA strands might cause distortions in the helical conformation of the DNA which are recognized by S1 nuclease *in vitro* and processed into DSBs (Kohfeldt *et al.*, 1988); however, molecular evidence is missing so far.

We are interested in the biological effects caused by SSSs in yeast cells. Gamma-induced damage of the nucleotide bases on opposite DNA strands are considered to be one type of DNA lesion responsible for cell death (Ward 1986). However, little is known about cellular repair and possible genetic and/or lethal effects of this type of gamma-induced DNA damage. This question seems very relevant as SSSs are by no means rare lesions. In contrast, they are approximately 1.5–2 times more frequent than DSBs in phage Lambda and yeast (Martin-Bertram *et al.*, 1983; Geigl and Eckardt-Schupp, 1990). It is well known that DSBs are efficiently repaired in yeast chromatin (for review, see Game, 1983; Luchnik *et al.*, 1977; Resnick, 1976; Resnick and Martin, 1976; Szostak *et al.*, 1983). Nevertheless, DSBs are regarded as the crucial, primary form of DNA damage leading to cell death (Frankenberg and Frankenberg-Schwager, 1981).

To monitor induction and repair of SSSs and DSBs we made use of pulsed-field gel electrophoresis (PFGE) (Geigl and Eckardt-Schupp, 1990), which allows the separation of large, chromosome-sized DNA molecules (for review, see Cantor *et al.*, 1988). Full-length chromosomal DNA of yeast migrates in the PFGE according to its length using a suitable switching time interval (Carle and Olsen, 1984). Each of the 16 chromosome species of yeast appears as a distinct band in the ethidium bromide-stained gel. If DSBs

are induced by *in vivo* gamma-irradiation alone or by subsequent *in vitro* treatment with S1 nuclease, shorter DNA molecules occur because of chromosomal breakage. Consequently, the intensity of the gel bands decreases and simultaneously a smear appears at the bottom of the gel caused by a heterogeneous population of smaller DNA molecules. If the cells have the opportunity to recover after irradiation before lysis and DNA preparation in agarose, repair processes lead to an increase in the number of originally-sized DNA molecules migrating as discrete bands and a decrease in broken DNA molecules migrating as a smear in the pulsed-field gel.

Using this technique we can show that SSSs are repaired as efficiently as DSBs in a repair-competent diploid yeast strain. Repair of DSBs and SSSs was analysed in individual chromosome species of *Saccharomyces cerevisiae* identifiable as distinct bands in the pulsed-field gels. We present results indicating that different chromosome species show different time courses for the repair of both DSBs and SSSs.

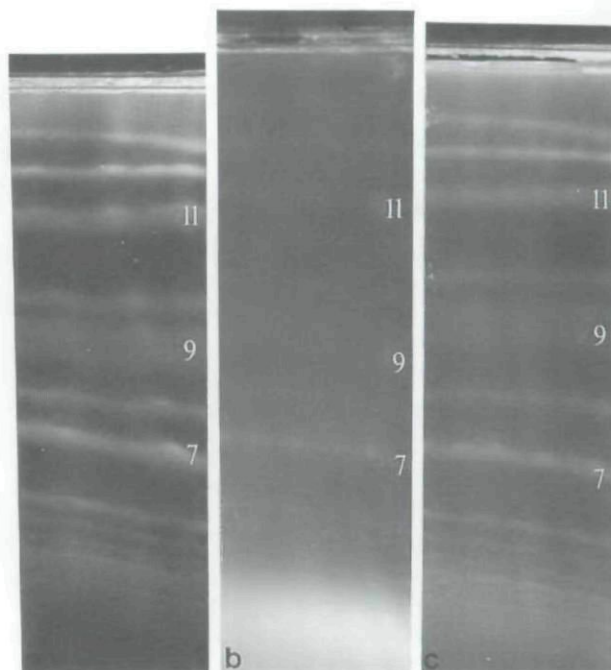

Fig. 1. Separation of BK0 cells irradiated with 200 Gy and treated with S1 nuclease.
a. Non-irradiated (= control).
b. Directly after irradiation (= induction of SSSs and DSBs; 0 hours of LHR).
c. After 48 hours of LHR (= repair of SSS and DSB).
The analysed double bands 7, 9, and 11 are indicated by the numbers 7, 9, and 11.

Results

In order to investigate the ability of *S. cerevisiae* to repair SSSs we irradiated cells of the diploid repair-proficient strain BK0 with [^{60}Co]-gamma rays under anoxic conditions in order to optimize the induction of SSSs by direct action of the radiation and to minimize strand breakage due to indirect radiation effects by hydroxyl radicals. After irradiation the cells were kept under non-growth conditions in phosphate buffer (pH 7) at 28°C for various time intervals up to 48 hours. These post-irradiation conditions ('liquid holding recovery', LHR) resulted in an increase in the fraction of treated cells that survive, as monitored by colony-forming ability (Patrick and Haynes, 1968). It was proved that DSBs are repaired efficiently in diploid repair-proficient yeast cells during LHR after irradiation (Frankenberg-Schwager *et al.*, 1980a,b; Frankenberg and Frankenberg-Schwager, 1981). Therefore we measured the number of induced DSBs per chromosome (as an internal control), and of induced SSSs per chromosome immediately after irradiation, and after various periods of repair under non-growth conditions.

At a dose of 200 Gy (under anoxic conditions), 60% of the cells survived upon direct plating on complete medium, and 90% survived when plated after 48 hours of liquid holding. The number of DSBs and of SSSs was measured in samples of [^{60}Co]-gamma-irradiated cells kept for 0, 4, 8, 12, 24 and 48 hours in phosphate buffer, embedded in agarose, and lysed enzymatically. One part was treated with S1 nuclease, and the other half was kept in buffer alone under identical conditions. Then the DNA of the S1-treated and non-treated samples was separated by PFGE. Preparation of the DNA, S1 nuclease-treatment, PFGE, evaluation of the gels, and calculation of the DSBs per molecule were done according to our standard protocol (Geigl and Eckardt-Schupp, 1990). By laser densitometry of the ethidium bromide-stained gels we evaluated the bands corresponding to the larger chromosomes only. The smaller chromosomes could not be evaluated without error because of comigration of the smear of broken DNA molecules.

Following this procedure we found that the DNA samples separated by PFGE showed increasing intensities of all bands in the gels for both S1-treated and non-treated samples with increasing time of buffer holding relative to the DNA derived from the gamma-irradiated cells without buffer holding (Fig. 1). This indicates an increase in the amount of high molecular, e.g. successfully repaired, chromosomal DNA molecules of every chromosome species. The numbers of DSBs and SSSs were calculated for the eight upper bands in the gel corresponding to the larger chromosomal DNA molecules of defined length. The numbers of DSBs and SSSs, respectively, calculated for the chromosomes migrating as double bands numbers 7, 9 and 11, for example, are shown in Fig. 2. As estimated by their length according to a standard strain (AB 972 , Carle and Olson, 1985) double

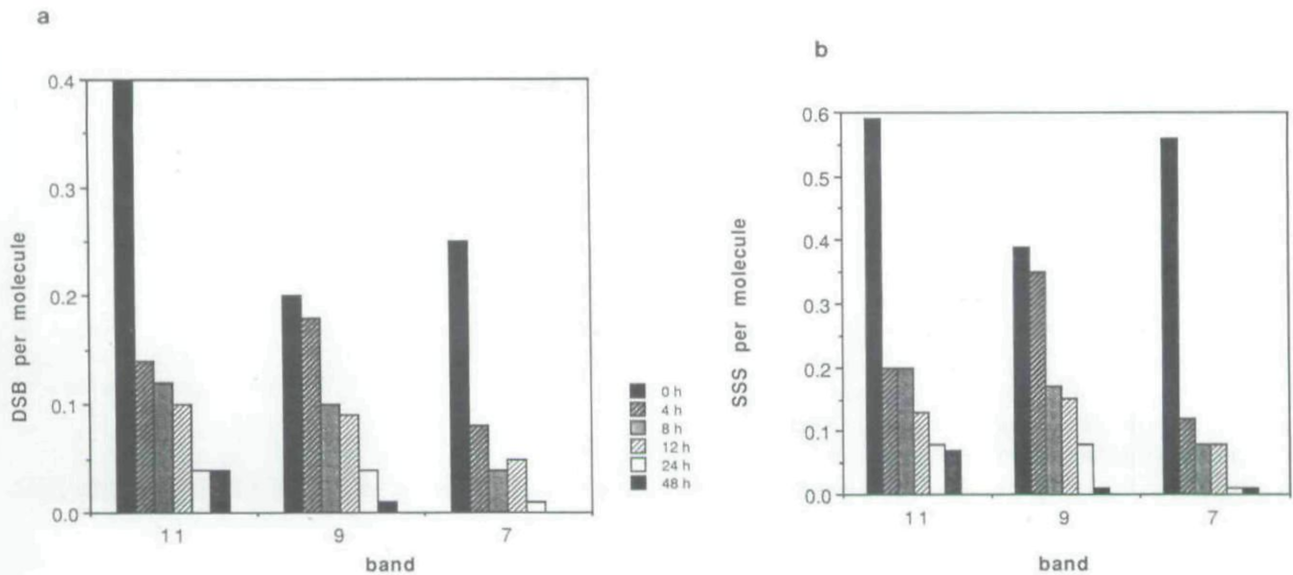

Fig. 2. Number of DSBs (a) and SSSs (b) per chromosomal DNA molecule of BK0 cells migrating as double bands 11, 9, and 7 in the PFGE after induction with 200 Gy *in vivo* [^{60}Co]-gamma irradiation and subsequent LHR for various times in hours (h).

band 7 represents probably the DNA of chromosomes V+VIII (about 600 kb), double band 9 chromosomes II+XIV (about 830 kb), and double band 11 chromosomes XIII+XVI (about 980 kb), respectively. Figure 2 depicts the decrease in DSBs (a) and SSSs (b) as a function of LHR time after the dose of 200 Gy of gamma rays was applied under anoxic conditions. It is obvious that DSBs are efficiently repaired under non-growth conditions, which is in good agreement with earlier findings by Frankenberg-Schwager *et al.* (1980a). Furthermore, they strongly support the notion that SSSs are repaired in diploid repair-proficient yeast strains as efficiently as DSBs.

Unexpectedly, the different chromosomes migrating as single, or at most double, bands in the PFGE showed distinguishable rather than similar kinetics of repair during post-irradiation buffer holding, which is obvious from the data shown in Fig. 2a and 2b. For example, about four fifths of the DSBs and SSSs induced in the chromosomal DNA migrating as band 7 have been removed within 4 hours, whereas this process took about 24 hours for the chromosomal DNA migrating in band 9 and little more than 12 hours in the DNA visible in band 11. The same features were found for the repair of DSBs as well.

Discussion

It had been our aim to analyse the induction and repair of SSSs in yeast chromatin which are induced by gamma rays under anoxic conditions nearly twice as frequently as DSBs (Andrews *et al.*, 1984; Geigl and Eckardt-Schupp, 1990).

Recently, we have developed a technique using PFGE for the quantification of DSBs in yeast chromosomal DNA (Geigl and Eckardt-Schupp, 1990). This new technique of DSB analysis has the advantage of detecting DNA damage converted *in vitro* by enzymatic processing to 'secondary DSBs', as for instance SSSs by S1 nuclease. Furthermore, it allows the analysis of DNA damage in individual chromosomes of yeast which cannot be visualized at any stage of the cell cycle because of insufficient condensation. PFGE of suitably prepared DNA from irradiated cells followed by densitometry of ethidium bromide-stained gels allows the calculation of the number of DSBs and SSSs for basically every one of the 16 chromosome species of *S. cerevisiae*, which is not possible with any of the conventional techniques like neutral sucrose sedimentation or elution. The new technique is sufficiently sensitive to identify as few as one DSB per five chromosomes. The DSB rates calculated per Gy and base pair using PFGE in different experimental approaches range between 1.45 and 3.3×10^{-9} (Geigl and Eckardt-Schupp, 1990; A. A. Friedl and F. Eckardt-Schupp, submitted). They are in good agreement with data obtained for yeast and mammalian cells using comparable conditions of irradiation but conventional techniques of DSB measurement (Frankenberg-Schwager *et al.*, 1979: 1.5×10^{-9} DSBs/Gy/base pair; Lennartz *et al.* (1975): 1.6×10^{-9} DSBs/Gy/base pair). The evaluation of ethidium bromide-stained pulsed-field gels has certain limitations. First, double bands with two chromosomal species of similar length cannot be distinguished, and second, bands representing smaller chromosomes are superimposed by

the smear of broken DNA molecules of the larger chromosomes. Using Southern hybridization techniques with chromosome-specific, radioactively labelled gene probes (Contopoulou *et al.*, 1987; Geigl and Eckardt-Schupp, 1990), this problem can be overcome.

We have examined induction and repair of SSSs induced by [^{60}Co]-gamma radiation by evaluating ethidium bromide-stained pulsed-field gels, and therefore we have analysed only the eight largest chromosomes. We assume that for these bands the error introduced by the superimposing smear due to breakage is negligible at the dose applied. Our data for all bands measured clearly indicate that SSSs can be repaired efficiently in repair-proficient diploid yeast under non-growth conditions. It is obvious that the time course of repair of SSSs is very similar to that for DSBs (compare Fig. 2a with 2b). This similarity of the kinetics of SSSs and DSB repair determined in several parallel experiments suggests similar repair mechanisms for both types of lesions. We have experimental evidence that repair of SSSs requires the capacity of the cells to recombine: SSS repair was not carried out in a haploid repair-competent yeast strain, and was abolished in a *rad50* diploid (E.-M. Geigl and F. Eckardt-Schupp, in press).

One result of our studies was surprising: the time course of repair of SSSs and DSBs in different bands corresponding to different chromosomes was significantly different. This effect is very obvious for bands 7, 9 and 11, but is also noticeable for bands corresponding to other chromosomes of the repair-competent strain BK0 as well as for the bands in the *rad3* diploid strain, BK3 (E.-M. Geigl and F. Eckardt-Schupp, in press) that are comparable to the bands 7, 9 and 11 of BK0. BK3 is competent in the repair of DSBs and SSSs. So far, we have no explanation and no further experimental analysis for the different kinetics of DSB and SSS repair in the various chromosomes of yeast. However, it might be taken as an indication that DSB and SSS repair does not occur uniformly throughout the genome.

This finding is in accordance with other findings that both induction of damage and effectiveness and velocity of repair of DNA damage may depend on the chromatin structure (Hagen, 1986; Oleinick *et al.*, 1983). Furthermore, there is evidence that repair differs in transcribed and non-transcribed genes in yeast (Terleth *et al.*, 1989; 1990) and mammalian cells (van Zeeland *et al.*, 1981; Bohr and Hanawalt, 1984; Reeves, 1984; Madhani *et al.*, 1986). Lesions are removed more rapidly from transcriptionally active genes than from bulk DNA (Mellon *et al.*, 1987), suggesting that microheterogeneity of DNA repair may be a general phenomenon (Bohr *et al.*, 1986). Cellular survival may be correlated with the ability to repair essential regions of the genome rather than with overall repair levels (Bohr and Hanawalt, 1987).

Application of PFGE on yeast and mammalian chromatin will allow us to pursue the question of the influence of chromatin structure on induction and repair of DNA damage and hence may offer a new approach to the question of the hierarchy of repair processes (Bohr and Hanawalt, 1987).

Experimental procedures

Strains of S. cerevisiae

The strain of *S. cerevisiae* used in this analysis generated as diploid derivative of the D7 line (Kunz and Haynes, 1982; Zimmermann *et al.*, 1975) has been shown to have the following genotype:

a ade2-40, cyh2, trp5-12, ilv1-92

α ade2-119, CYH2, trp5-27, ilv1-92

BK0 is a diploid wild-type homozygous with respect to DNA repair genes (*RAD/RAD*).

Media, culture, and irradiation conditions

Culture of yeast cells and [^{60}Co]-gamma irradiation was carried out as described elsewhere (Geigl and Eckardt-Schupp, 1990). To allow the irradiated cells to recover under non-growth conditions (LHR), no EDTA was added after irradiation (as was done for the control) and the cells were gently shaken in 0.1 M phosphate buffer (pH 7.0) at a cell density of 2×10^9 cells per ml in a water bath at 28°C.

DNA preparation, S1 nuclease treatment, and PFGE

The cells were embedded and lysed in agarose (3.7×10^8 cells per 150 μl block). Each cell sample gave about 10 blocks. The blocks were melted and treated with 1 U nuclease S1 per 150 μl block at 37°C and pH 4.5 for 30 min in S1 buffer (3.3 mM sodium acetate, 0.2 M sodium chloride, 3 μM zinc sulphate). Two samples, one treated with S1 nuclease and one without, were loaded onto a 1% agarose gel and separated by PFGE as described (Geigl and Eckardt-Schupp, 1990).

Evaluation of the photonegatives of the gels

Agarose blocks were made from all cell samples, i.e. cells that were not irradiated (called 'control' in Fig. 1), irradiated cells (= '0 hour' value in Figs 1 and 2), and cells irradiated and held in phosphate buffer for various time intervals (= '4–48 hours' values in Figs 1 and 2). For statistical reasons three parallel experimental series were carried out independently. Each cell sample was subjected to PFGE three times. This experimental design resulted in nine gel photos for each type of cell sample showing the effects of the treatments without and with S1 nuclease in the two lanes. Three laser-densitograms were taken from each photonegative of the ethidium bromide-stained gels at three different positions of each lane. Evaluation of the resulting 27 densitograms per cell sample and calculation of the number of DSBs and SSSs per molecule were carried out exactly according to Geigl and Eckardt-Schupp (1990). The average of the 27 values for the number of DSBs and SSSs (normally with deviations of 10–20%) obtained by this evaluation procedure for each experimental unit is given as a single bar in Fig. 2.

Chemicals

Low melting-point (Sea Plaque) and LE agarose were from FMC, and S1 nuclease and restriction enzymes from Boehringer Mannheim. Zymolyase was obtained from Miles, proteinase K from Serva, and phenylmethylsulphonyl-fluoride (PMSF) from Sigma.

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