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Chromosome-specific identification and quantification of S1 nuclease-sensitive sites in yeast chromatin by pulsed-field gel electrophoresis

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Summary

Sites that are sensitive to the single-strand-specific endonuclease S1 ('S1-sensitive sites', SSS) occur in native chromatin and, like DNA double-stranded breaks (DSB), they are induced by DNA-damaging agents, such as ionizing radiation. We have developed a method to quantify SSS and DSB in yeast chromatin by using pulsed-field gel electrophoresis (PFGE) to separate the intact chromosomal-length DNA molecules from the lower molecular-weight broken ones. Direct evaluation of the photonegatives of the ethidium bromide-stained gels by laser densitometry enabled us to calculate the numbers of DSB and SSS per DNA molecule. These numbers were determined from the bulk of the non-separated genomic DNA of yeast, corresponding to a single band in the PFGE (pulse time 10 seconds), and in each of the eight largest yeast chromosomes, corresponding to distinct bands in the PFGE gels (pulse time 50 seconds), which were not superimposed by the smear of the broken, low molecular-weight DNA. Furthermore, the induction of DSB and SSS in a specific chromosome (circular chromosome III) was determined by Southern hybridization of the PFGE gels with a suitable centromere probe, followed by densitometry of the autoradiographs. Our method allows the chromosome-specific monitoring of DSB and all those DNA structures that are processed either *in vivo* or *in vitro* into DSB and which may not be distributed randomly within the genome.

Introduction

In native chromatin, regions known as nuclease hypersensitive sites represent a small, highly selective fraction of the genome. These sites, which presumably vary in molecular structure, have been defined operationally by their pronounced sensitivity to nuclease cleavage. Generally, they represent 'open' *cis*-acting DNA sequences involved in the regulation of chromatin structure and function (for a review, see Gross and Garrard, 1988). In *Saccharomyces cerevisiae*, hypersensitive sites occur around centromeres, replication origins, recombination sequences, promoter elements, silencers, upstream activating sequences, presumptive transcription terminators, etc., as reviewed by Gross and Garrard (1988). These authors distinguish between two main categories of hypersensitive sites: constitutive and inducible ones. The presence of constitutive hypersensitive sites is described as normally preceding transcriptional activation and is thought to be independent of gene expression. Inducible sites are also found to appear prior to transcription but often persist long after removal of the inducing agent and/or continued transcription (Gross and Garrard, 1988).

All hypersensitive sites are determined by their underlying or flanking non-B-DNA conformation (Gross and Garrard, 1988). DNase I (Wu, 1980), as well as S1 nuclease, have been useful tools in detecting non-B-DNA structures, such as cruciforms, junctions between B- and Z-DNA, polypurine–polypyrimidine stretches and pyrimidine-rich sequences (Htun *et al.*, 1984; McKeon *et al.*, 1984; Mace *et al.*, 1983; Nickol and Felsenfeld, 1983). These sequences are non-randomly dispersed in the DNA of a variety of sources (Pulleyblank *et al.*, 1985; Goding and Russell, 1983), and presumably they contain single-stranded DNA, staggered single-stranded loops or other DNA conformations different from helical B-DNA (Htun *et al.*, 1984; Evans *et al.*, 1984; McKeon *et al.*, 1984; Mace *et al.*, 1983). Secondary structures that are potentially unstable in naked DNA might be stabilized by interaction with non-histone proteins in chromatin (Goding and Russell, 1983; Gross and Garrard, 1988) or by supercoiling in plasmids (Larsen and Weintraub, 1982).

The mechanisms leading to the formation of hypersensitive sites, as well as their function, are not fully understood. Because of the diversity of biological functions associated with hypersensitive sites, different mechanisms are likely to occur. In some cases, interaction of DNA sequences with trans-acting proteins (such as

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topoisomerase I and II, transcription factors, RNA polymerase II) is involved. DNA methylation, torsional stress, and non-B-DNA are also considered to be of importance (for review, see Gross and Garrard, 1988).

In addition to the enhanced sensitivity to various nucleases, hypersensitive sites are also more sensitive to u.v. light and chemical carcinogens, which specifically act with DNA (Elgin, 1982; McCormick *et al.*, 1983; Gross and Garrard, 1988; Kohwi-Shigematsu *et al.*, 1988). S1 nuclease-sensitive sites (SSS) can be induced by ionizing radiation in phage DNA (Martin-Bertram, 1981; Martin-Bertram and Hagen, 1979; Martin-Bertram *et al.*, 1983; 1984) and in the chromatin of yeast (Andrews *et al.*, 1984) and mammalian cells (Paterson *et al.*, 1976; Paterson, 1978; Yoshizawa *et al.*, 1976; Furuno *et al.*, 1979). The molecular structure of these inducible hypersensitive sites and their relationship to constitutive sites are unknown. So far, it has been assumed that these radiation-induced S1-sensitive sites consist of stretches of damaged neighbouring bases ('clustered base damage' or, according to Ward (1985), 'locally multiply damaged sites', LMDS), which supposedly are reduced in their ability to pair and which function as a substrate for S1 nuclease. However, any biochemical or molecular evidence is missing so far.

In view of the specific interaction of numerous DNA-damaging agents with hypersensitive sites (for review, see Gross and Garrard, 1988), we posed the question as to whether ionizing radiation-induced SSS necessarily represent locally multiply damaged DNA regions, which are expected to be distributed at random in the genome like gamma-induced DNA double-strand breaks (DSB) (Frankenberg *et al.*, 1981) or whether ionizing radiation interacts specifically with regions exhibiting the potential for hypersensitive site formation. If this were the case, SSS induced by ionizing radiation would not be expected to be distributed randomly in the genome. We considered this point to be very important for understanding the biological function of ionizing radiation-induced SSS, which is completely unknown to date.

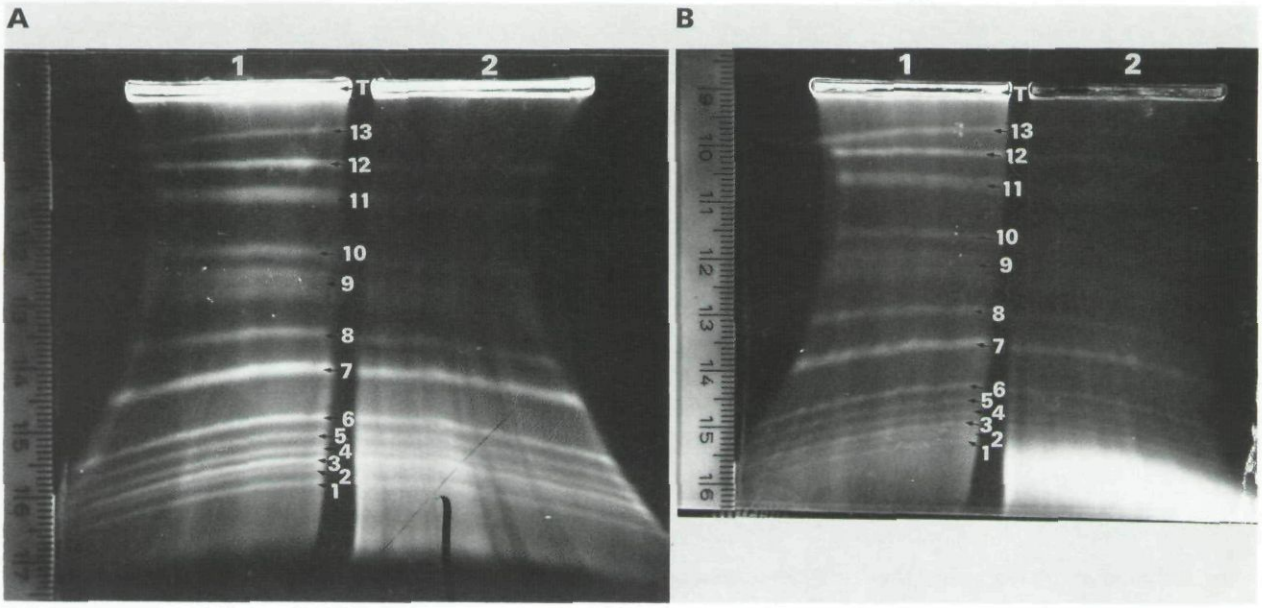
Our long-term goal is to investigate the biological relevance of the radiation-induced S1-sensitive regions in yeast chromatin, including their distribution, molecular structure and potential accessibility for DNA repair processes, and finally, their possible importance for the induction of genetic and epigenetic events.

In order to approach these questions, we had to develop a sensitive and reliable method to quantify and, possibly, localize SSS in yeast chromatin (Geigl *et al.*, 1986). For this purpose, we established a method for using pulsed-field gel electrophoresis (PFGE) to analyse breaks in DNA of very high molecular weight. PFGE allows separation of full-length chromosomal yeast DNA so that each band in the agarose gel corresponds to a yeast chromosome (Carle and Olson, 1984; Schwartz and Cantor, 1984). The degradation of the chromosomal, high molecular-weight DNA, either induced by ionizing radiation *in vivo* (Frankenberg-Schwager *et al.*, 1979; Resnick and Martin, 1976) or originating from SSS by treatment with S1 nuclease *in vitro*, could be visualized and quantified as a decrease in the intensity of each band in the PFGE gel and a corresponding increase in the smear representing the broken, low molecular-weight DNA. In order to avoid any unspecific DNA degradation by shearing, we embedded the yeast cells in agarose, which allows all enzymatic treatments to be carried out under buffer-like conditions (Geigl, 1987). Owing to the extremely cautious handling of the DNA, this method allows the detection of as few as 0.1–0.2 DSB per yeast chromosome. Furthermore, this method is applicable for the quantification of DSB and SSS in individual chromosomes as well as to all DNA structures that are processed into DSB *in vivo* or *in vitro*.

Results

Identification of SSS in yeast chromatin

Using PFGE, the presence of SSS in the DNA of untreated and [^{60}Co]-gamma-irradiated yeast cells can be visualized by the enhanced chromosomal breakage, e.g. the occurrence of DSB caused by the S1 nuclease treatment. We have analysed stationary cells of the diploid yeast strain BK0 after applying increasing doses of [^{60}Co]-gamma rays (0, 100, 200, 400, 800 Gy). Following irradiation, a small sample was taken to monitor survival, which varied between 100% (0 Gy) and 40% (800 Gy), indicating the dose range of biological significance. Each sample was divided into two aliquots: one was treated with S1 nuclease, and the other was incubated in the same buffer without S1 nuclease. Both samples were analysed by PFGE. As an example, Fig. 1 shows the PFGE separation of a non-irradiated control and a sample irradiated with 200 Gy with or without S1 nuclease treatment. The corresponding densitogram compares the non-irradiated control DNA with the irradiated DNA. An irradiation-induced decrease in the intensities of the individual bands and a corresponding increase in the intensity of the smear attributed to [^{60}Co]-gamma-induced DSB in the chromosomal DNA are evident. The additional increase in DNA breakage caused by S1 nuclease treatment can be seen clearly by comparing the left and right lanes of both gels. The breakage induced by S1 nuclease in untreated control cells was significant, indicating the presence of hypersensitive regions in native chromatin. The extent of S1 nuclease-dependent breakage in the irradiated samples was considerably larger than in the non-irradiated control. The evaluation of the photonegatives of gels obtained from various samples treated with increasing doses of radiation (Geigl, 1987) strongly supports the notion of a



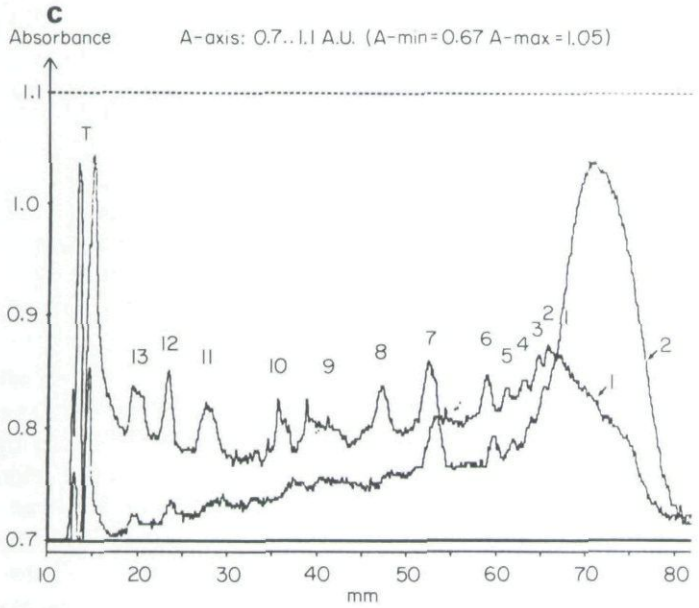


Fig. 1. Separation of the individual chromosomes of the diploid yeast strain BK0 in the PFGE with a switching interval of 50 seconds. Cells have been lysed without irradiation (A) and after irradiation with 200 Gy (B). Agarose cell lysates were incubated under standard conditions without (1) and with (2) S1 nuclease. The densitogram (C) was made from the gel photo (B).

clear linear increase in S1 nuclease-mediated DNA breakage above the spontaneous background (Geigl *et al.*, 1986). It should be pointed out that although these sites in untreated and treated chromatin are defined by their sensitivity towards S1 nuclease, it is unknown whether they represent similar molecular structures.

Quantification of SSS

In order to quantify the dose-dependent occurrence of DSB and SSS, respectively, we adopted three different approaches.

First, we evaluated a series of PFGE gels of DNA obtained from non-treated and [^{60}Co]-gamma-treated (200 Gy) cells using switching intervals of 10 seconds. Short pulses of 10 seconds do not separate intact yeast chromosomal DNA molecules, but rather yield a single band containing all chromosomal DNA molecules in the upper part of the gel. The heterogeneous population of broken molecules is visible as a smear in the lower part of the gel (Fig. 2). S1 treatment increased the relative amount of DNA in this smear. This experimental design allowed the quantification of DSB and SSS in the total yeast genome (1.4×10^7 base pairs per haploid genome), using the direct evaluation procedure of the bands described in the *Experimental procedures*. From this, the number of SSS in

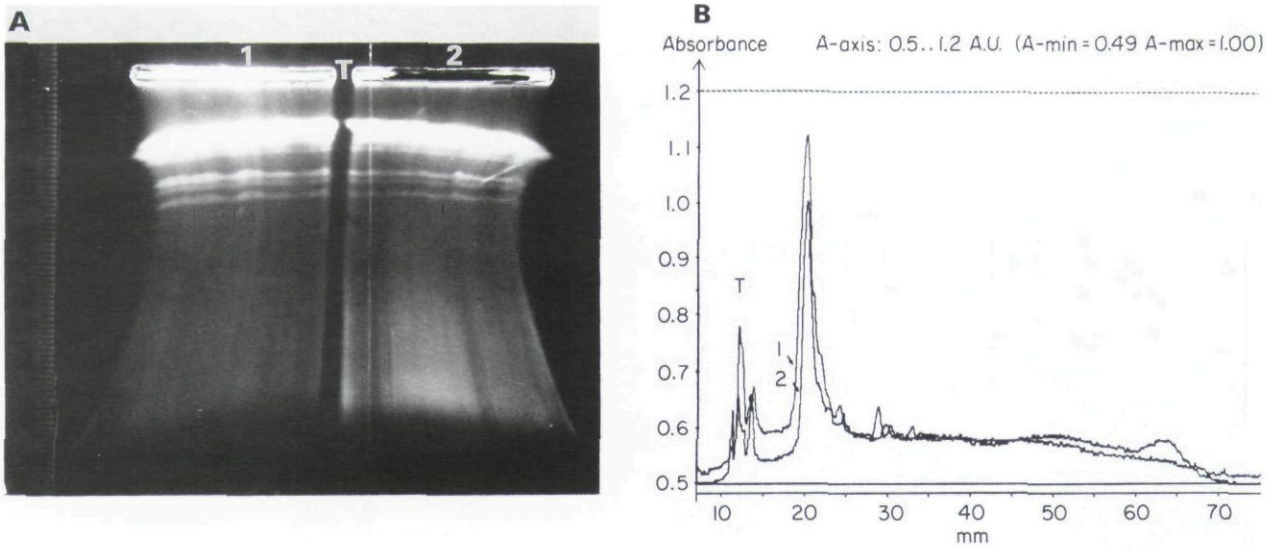


Fig. 2. DSB analysis of the total genome of the strain BK0 irradiated with gamma-rays (200 Gy) under anaerobic conditions. The agarose lysates of the cells were incubated under standard conditions without (1) and with (2) S1 nuclease and separated in the PFGE by a switching time of 10 seconds. The compression of the unbroken chromosomal DNA molecules in the gel (A) is seen as one large and some small peaks, whereas the smear caused by the broken DNA molecules in the gel (A) appears as a 'plateau' in the densitogram (B).

untreated chromatin of stationary-phase yeast cells (by correlating the non-irradiated controls with and without S1 treatment) and the numbers of DSB and SSS induced by 200 Gy per average chromosomal DNA molecule of 800 kbp (1/16 of the total genome) were calculated (Table 1).

Second, we determined the number of DSB and SSS in individual chromosomes. We separated the genome of the non-irradiated and irradiated (200 Gy) cell samples using switching intervals of 50 seconds in the PFGE. This pulse time allows the separation of most of the 16 yeast chromosomes that migrate as single bands (Fig. 1). The evaluation of the photonegatives enabled us to calculate the number of DSB and SSS for each PFGE band. Figure 3 shows the number of gamma-induced DSB and SSS per DNA molecule for bands 6–13. From these numbers, the average of 0.23 ± 0.14 and 0.43 ± 0.19 SSS per average chromosomal DNA (1000 kbp as average length of the eight largest chromosomes) induced by 200 Gy was calculated (Table 1). The number of SSS occurring in untreated DNA was determined as about 0.14 per average chromosomal DNA molecule (1000 kbp) by comparing the S1-treated with the non-treated, non-irradiated cell samples (Table 1).

Table 1. Number of SSS and DSB per DNA molecule, induced by [^{60}Co]-gamma rays (200 Gy) under anaerobic conditions in diploid cells of *S. cerevisiae* and determined by PFGE using three different approaches.

	Approach	1	2	3
	Average size of the analysed DNA molecule	800 kbp	1000 kbp	350 kbp
Spontaneous SSS	SSS per approach-specific DNA molecule	0.08	0.14	0
	SSS per 10^7 base pairs	1.0	1.4	0
Gamma-ray-induced SSS (200 Gy)	SSS per approach-specific DNA molecule	0.62	0.43 ± 0.19	0.33
	SSS per 10^7 base pairs	7.78	4.3	9.43
Gamma-ray-induced DSB (200 Gy)	DSB per approach-specific DNA molecule	0.23	0.23 ± 0.14	0.23
	DSB per 10^7 base pairs	2.88	2.3	6.57

1 = 'average' chromosome (equivalent to 1/16 of the total genome).
2 = 'average' chromosome (equivalent to 1/8 of the 8 largest chromosomes).
3 = chromosome III (deletion due to circularization not considered).
Standard deviations of three independent determinations were calculated for approach 2. The data obtained by approaches 1 and 3 were both the results of one single experiment.

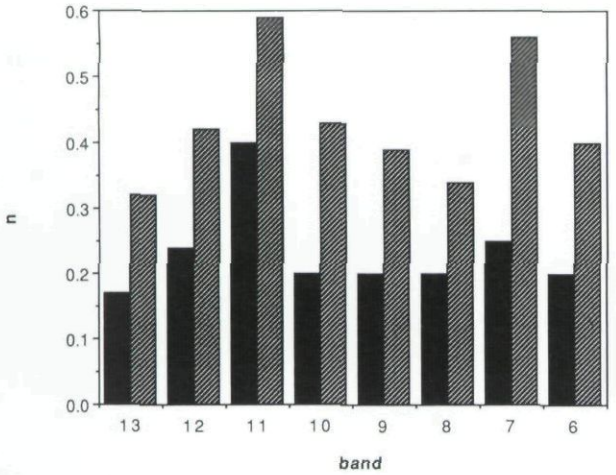


Fig. 3. Band-specific numbers of DSB and SSS per DNA molecule (*n*) induced in the diploid repair-proficient wild-type BK0 by gamma rays (200 Gy), as determined for the largest chromosomes numbered 6–13 in Fig. 1. PFGE was run with switching intervals of 50 seconds. DSB are shown as solid columns, and SSS as hatched columns.

Third, we measured the induction of DSB and SSS in an individual chromosome, chromosome III, using a diploid yeast strain with one circular and one linear chromosome III (Haber *et al.*, 1984). Circular DNA molecules of chromosomal length do not enter the gel during the PFGE (Hightower *et al.*, 1987). Linearization of the circular molecule by introduction of one DSB enables migration during PFGE to a position corresponding to its length (350 kb for chromosome III). Induction of more than one break per molecule results in a smear located below the distinct band of intact chromosome III molecules. The positions of the linearized chromosome III and the smear in the gel were visualized by Southern hybridization with the plasmid YeCen3-13, containing a centromeric sequence of chromosome III (McGrew *et al.*, 1986) (Fig. 4). Our method makes use of probing PFGE blots with suitable gene probes, such as the one described by Contopoulou *et al.* (1987). However, whereas these authors measured DSB in individual genes, we measured DSB and SSS in an individual chromosome of approximately 350 kbp length (Carle and Olson, 1985). The evaluation of the PFGE autoradiographs (Fig. 4) resulted in linear dose-effect curves for both DSB and DSB + SSS (Fig. 5). At 200 Gy, 0.23 DSB and 0.33 SSS are induced per chromosome III (Table 1). In order to compare the data obtained in chromosome III with those obtained by densitometry of the gel photonegative, the different chromosome size has to be taken into account.

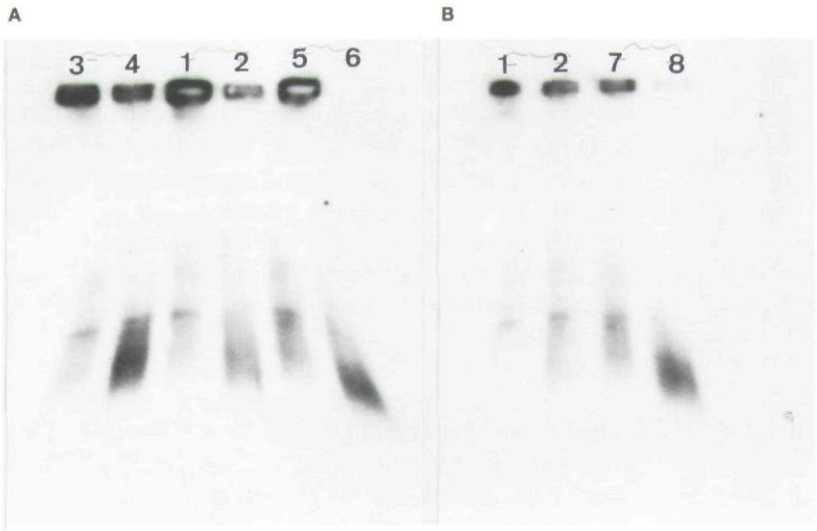
Discussion

We have used pulsed-field gel electrophoresis (PFGE) to identify and quantify S1-sensitive sites (SSS) in chromatin of non-irradiated and [^{60}Co]-gamma-irradiated yeast cells. Nuclease hypersensitive sites in native chromatin represent regions of local alterations of the superhelical DNA conformation, causing reduced or abolished nucleosome phasing (Gross and Garrard, 1988). In contrast, the molecular structure of SSS, which has been identified in the DNA of ionizing radiation-treated phage and prokaryotic and eukaryotic cells, is unknown. Despite their possible molecular differences, both types of structures are processed *in vitro* by nuclease S1 into double-strand breaks (DSB).

DSB can be quantified by various techniques. Conventional analytical ultracentrifugation and gel electrophoresis are restricted to small molecules and are therefore not applicable to DSB analysis of eukaryotic DNA at low, biologically relevant doses of radiation or of chemicals inducing DSB. Other sensitive techniques for analysing DSB, such as neutral sucrose gradient centrifugation (Frankenberg *et al.*, 1981; Frankenberg-Schwager *et al.*, 1979; Resnick and Martin, 1976), alkaline elution techniques and alkaline unwinding techniques (Kohn, 1979), require *in vivo* labelling of the DNA. Thus, this technique can only be applied to organisms that are able to take up radioactive labelled DNA precursors. They also restrict the use of enzymes to those functioning at a neutral or alkaline pH. However, the optimum of the nuclease S1 is pH 4.5.

Largely as a result of this last restriction, we had to establish new experimental conditions to detect and quantify regions sensitive to S1 nuclease in gamma-irradiated and non-irradiated yeast chromatin. The procedure which we have described allows quantification of DSB induced directly by ionizing radiation or derived by *in vitro* S1 nuclease treatment of SSS. The method detects as few as 0.1–0.2 DSB individually for each of the larger yeast chromosomes, whereas all other techniques allow determination of damage in the total genome only. In contrast to the evaluation presented by Contopoulou *et al.* (1987), which requires Southern hybridization with gene probes, we are able to determine the number of SSS and DSB per average chromosomal molecule by directly monitoring the intensity of the ethidium bromide-stained bands in the pulsed-field gels.

In order to prove the reliability of our method, we have used three different approaches to quantify DSB and SSS. First, by using PFGE with a short pulse time of 10 seconds, we have separated the smear of broken DNA molecules from the unbroken but otherwise unseparated chromosomal DNA, and determined the number of DSB and SSS per average-sized DNA molecule in the total genome. Second, we have quantified DSB and SSS chromosome specificity in the eight largest chromosomes from PFGE gels (pulse time 50 seconds) with well-separated, distinct chromosome bands not superimposed by the smear of



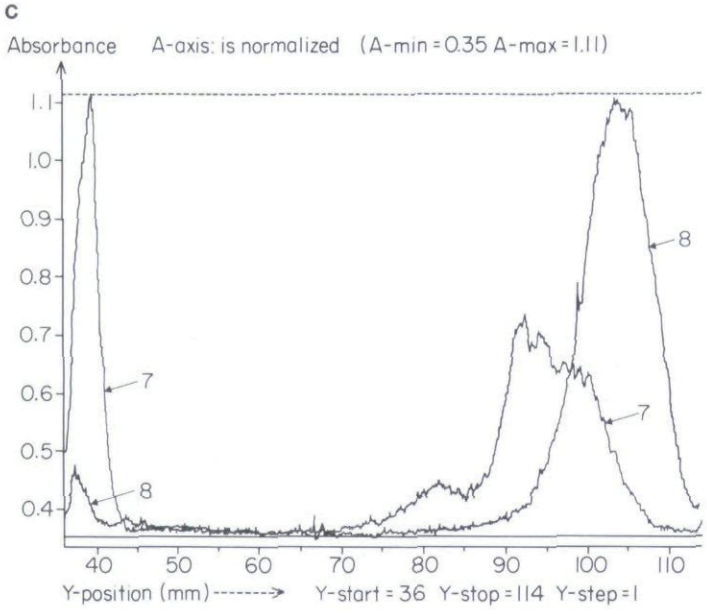


Fig. 4. Autoradiography of pulsed-field gels (50-second pulses) run with agarose lysates of the strain JH1030 with one circular chromosome III (A and B). The cells were irradiated with 0 Gy (1, 2), 200 Gy (3, 4), 400 Gy (5, 6) and 600 Gy (7, 8) and incubated under standard conditions without (1, 3, 5, 7) and with (2, 4, 6, 8) S1 nuclease. The densitogram (C) shows lanes 7 and 8.

randomly broken DNA. And third, we have measured the induction of DSB and SSS in one particular chromosome, the circular chromosome III. From Table 1 it is apparent that the three different types of measurements are in good agreement: 2.9–6.6 $\times 10^{-7}$ DSB per base pair and 4.3–9.5 $\times 10^{-7}$ SSS per base pair are induced at a dose of 200 Gy, corresponding to 80% survival. Relating the DSB induction rate to the average sized yeast chromosome, 1.8 $\times 10^{-9}$ DSB/Gy/base pair are induced. This value is in good agreement with the value of 1.5 $\times 10^{-9}$ DSB/Gy/base pair which has been obtained by neutral sucrose-gradient sedimentation (Frankenberg *et al.*, 1981; Frankenberg-Schwager *et al.*, 1979; Resnick and Martin, 1976). The induction rate of SSS by ionizing radiation has not been measured in yeast so far. We are, however, confident that the rates for the induction of SSS are reliable using the DSB measurements as an internal control. Applying the same evaluation technique, we determined the number of SSS in non-irradiated cells as 1.2 $\times 10^{-7}$ per nucleotide. Irradiation of cells with 200 Gy elevates the frequency of SSS to 7.17 $\times 10^{-7}$ per base pair (average), which is approximately the 1.6-fold DSB induction rate. In lambda

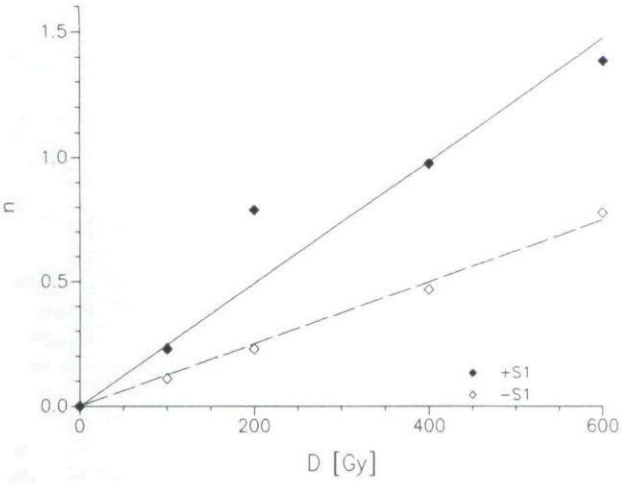


Fig. 5. Dose-response curve for the induction of DSB per molecule (open symbols) and SSS + DSB per molecule (closed symbols) induced in chromosome III for [^{60}Co]-gamma rays, as evaluated from the autoradiographs shown in Fig. 4. The gradients represent the regression of the single points. The number of SSS is calculated as the difference in the numbers of DSB + SSS and DSB only.

phage DNA gamma-irradiated under anoxic conditions, SSS were determined as occurring twice as often as DSB (Junker *et al.*, 1984).

The chromosome-specific rates of DSB and SSS induced at a dose of 200 Gy and calculated for the eight bands representing the larger yeast chromosomes are shown in Fig. 3. Comparable rates of DSB per DNA molecule are found for bands 6–13 (with the exception of band 11), whereas the rates of SSS per DNA molecule show much larger variation. Similar results have been obtained with other diploid yeast strains (Geigl, 1987), which supports the notion of a non-homogeneous distribution of SSS in the yeast genome. Further support comes from indirect end-labelling experiments carried out for the yeast genes *CEN4*, *CEN6*, *ADH2*, *ADH4* and the non-expressed *PHO5* gene (Geigl, 1987). At various doses of gamma-irradiation, no induced SSS were identified, although DSB were measured at the expected frequencies. However, using the same technique, SSS could be identified in the *PHO5* region under conditions which favoured transcription of the gene (Geigl, 1987). These findings indicate that ionizing radiation-induced SSS (as opposed to randomly distributed DSB) seem to represent non-randomly distributed structures in yeast chromatin. Further indications for a non-statistical distribution of SSS come from *in vitro* studies of dry crystalline DNA (Baverstock, 1985) and *in vivo* analysis of mammalian DNA (Oleinick *et al.*, 1983). Assuming a non-random distribution of ionizing radiation-induced SSS (though the experimental proof is still pending) the question arises as to whether SSS are induced preferentially in genomic regions of DNA structures that favour hypersensitive site formation.

It has been suggested that hypersensitive sites in native chromatin represent 'hot spots' for genetic processes like homologous recombination, gene conversion and mutation (Hentschel, 1982; Larsen and Weintraub, 1982; Gross and Garrard, 1988). Hypersensitive sites appear to be preferred targets for the integration of retroviruses (Rohdewold *et al.*, 1987) and possibly for deletions and translocations as well (Gross and Garrard, 1988). Thus, since SSS are induced by ionizing radiation in yeast chromatin more frequently than DSB, it appears very important to investigate the biological function of ionizing radiation-induced SSS, especially their genetic relevance.

In conclusion, we have shown that pulsed-field gel electrophoresis is a suitable new method for detecting and quantifying DSB in the yeast genome. In addition, spontaneous and induced SSS converted into DSB *in vitro* can be detected with high sensivity for individual yeast chromosomes. Using suitable gene probes, this method provides a new approach for investigating the distribution of DSB and SSS in different regions of the chromosomes, as well as their accessibility for repair. We propose that this technique is applicable to the analysis of all DNA damage convertible into DSB, as well as to other eukaryotic cells.

Experimental procedures

Strains of Saccharomyces cerevisiae

The diploid strain BK0, kindly donated by Dr B. A. Kunz, Winnipeg, Canada (Kunz, 1981, PhD thesis, York University, Toronto; Kunz and Haynes, 1981) and the diploid strain JH1030, containing one circularized chromosome III, constructed and kindly donated by Dr J. Haber (Haber *et al.*, 1984), were used for this analysis.

Plasmids

Plasmid YeCen3-13 Chr III, which contains a 624 bp sequence of the centromere region (McGrew *et al.*, 1986), was used in Southern hybridization to identify chromosome III.

Media, culture and irradiation conditions

Yeast cells were grown to stationary phase at 28°C in YPD medium (1% yeast extract, 2% Bacto peptone, 2% glucose; Difco), harvested by centrifugation, washed twice in KH_2PO_4/K_2HPO_4 buffer (0.1 M, pH 7.0) and resuspended in the same buffer at a concentration of 2×10^9 cells ml^{-1}. The cell suspension was kept on ice and de-aerated by bubbling with nitrogen for about 30 min. The anaerobic conditions minimized indirect effects of hydroxy radicals resulting in predominantly DNA single-strand breakage, and therefore reduced single-strand break-caused smears on the gels. Irradiations were performed on ice in the presence of nitrogen, as described by Andrews *et al.* (1984), using

a [^{60}Co]-gamma source (Atomic Energy of Canada Limited) at a dose rate of 63 Gy min^{-1}. After irradiation, a sample was taken to determine survival by plating suitably diluted aliquots on YPD plates. Immediately afterwards, EDTA was added, to a concentration of at least 0.1 M, to the cell suspension to be analysed by PFGE and kept on ice.

DNA preparation

After irradiation, the cells were washed twice with 50 mM EDTA (pH 7.5) and resuspended in 50 mM EDTA to a concentration of 1 × 10^{10} cells in 1.5 ml suspension. 0.5 ml SEC buffer (1 M sorbitol, 0.1 M EDTA, 0.01 M citrate, phosphate buffer pH 7.0) and 10 mg zymolyase 20000 were added. The cell suspension was mixed with 2 ml 1% low melting-point agarose (Sea Plaque) in 0.125 M EDTA (pH 7.5) at 42°C, distributed in a 150 μl agarose block maker and cooled on ice. The 150 μl agarose blocks were removed from the block maker and incubated in 5 ml 0.5 M EDTA, 0.01 M Tris-HCl, pH 8.0, at 37°C for 1 h. After spheroblast induction the agarose blocks were transferred to 5 ml 0.5 M EDTA (pH 9.0), 0.01 M Tris-HCl (pH 8.0), 1% N-lauroylsarcosine, 0.1% proteinase K and incubated overnight at 50°C. On the next day, the agarose blocks were then rinsed at 4°C for 2–3 days with 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1 mM phenylmethylsulphonylfluoride (PMSF) and the buffer changed several times. The agarose blocks were stored at 4°C in the same PMSF-containing buffer. These blocks contained full-length chromosomal DNA accessible for digestion with restriction and other enzymes. The DNA content per lysate block was estimated from the cell titre of the cell suspension.

Nuclease S1 treatment

Agarose blocks containig 10 μg DNA were equilibrated in 1 ml of S1 buffer at pH 4.5 for 1 h, melted at 65°C and incubated for 5 min at 37°C. Treatment with nuclease S1 was carried out at 37°C for 30 min in 3.3 mM sodium acetate, 0.2 M sodium chloride, 3 μM $ZnSO_4$, pH 4.5 at a concentration of 1 U nuclease S1 for 1 μg DNA. The reaction was stopped with 20 mM EDTA and 2 M Tris base and then the liquid agarose mix was poured into the slots of a 1.5% agarose gel in 0.4 × TBE (36 mM Tris-HCl, 36 mM sodium borate, 8 mM EDTA).

Under these conditions, the S1 nuclease activity exhibited the same activity in melted agarose as in aqueous solution. (i) Single-stranded M13-DNA in agarose was completely digested by 30 U μg^{-1} or more nuclease S1, whereas double-stranded lambda DNA in agarose was not degraded by nuclease S1 up to 50 U μg^{-1} DNA. In contrast, S1 nuclease degraded partially heat-denatured genomic yeast DNA at a concentration of 1 U μg^{-1} DNA in agarose (Geigl, 1987). (ii) S1 nuclease did not cleave the strand opposite DNase I- or gamma-induced single-strand breaks (SSB). Neither DNase I-nicked pBR322 plasmid DNA (open circle carrying 1 SSB) nor plasmid DNA irradiated with increasing doses of gamma-rays were degraded by 1 U nuclease S1 μg^{-1} DNA. This was demonstrated by comparing S1-treated DNA with heat-denatured DNA in a native agarose gel (Geigl, 1987). (iii) S1 nuclease activity was not inhibited by the proteinase K inhibitor PMSF, as shown with nuclease S1-digested partially heat-denatured lambda DNA or single-stranded M13-DNA in the presence and absence of 1 mM PMSF (Geigl, 1987).

Pulsed-field gel electrophoresis

We used a special type of PFGE called Orthogonal Field Alteration Gel Electrophoresis (OFAGE) performed in an apparatus constructed according to Carle and Olson (1984). To compensate for the deformation of the lanes in the OFA gels, we chose a symmetrical arrangement with only two samples per gel. This arrangement ensures that the corresponding bands in the two lanes show identical extension (width and height) and migration distances, which facilitates the quantification of the densities of the bands (Geigl, 1987). The gels were run at 300 V, 200 mA for 18 h with a switching interval of 10 or 50 s. The gels were stained in 300 ml 0.4 × TBE containing 30 μl ethidium bromide solution (1 mg ml^{-1}) for 1 d, and destained in 0.4 × TBE for another day.

For Southern transfer, the gels were shaken in 0.25 N HCl for depurinization (40 min), in 0.5 N NaOH, 1.5 M NaCl for denaturation (30 min) and, finally, in 1 M ammonium acetate and 0.02 N NaOH for neutralization (20 min). The DNA was transferred to a nitrocellulose membrane (Schleicher and Schuell) in 1 M ammonium acetate and 0.02 N NaOH. Southern hybridization was carried out according to Southern (1975).

Evaluation of the photonegatives of the gels

The photonegatives of the gels (Polaroid film type 665) were scanned with an LKB laser densitometer (Ultrascan XL). To account for irregularities in the DNA content within a single band of a gel, three densitograms were taken from one lane, which allows obvious 'breakaways' to be eliminated. The relative area of a single peak in a densitogram corresponds to the amount of unbroken DNA of a specific chromosome, which appears as a distinct band in the gel. For each chromosome band, the value of its specific relative area was correlated to the total DNA per lane determined from the areas of all peaks. The average of the equivalent peaks of three densitograms per lane of an S1-treated and/or irradiated sample was divided by the average value obtained from three densitograms of the non-irradiated control sample, resulting in the ratio 'q'. From the negative natural logarithm of the ratio 'q' the number of breaks per molecule, 'n', was calculated according to the equation $n = lnq$ (Jacobs *et al.*, 1972; Kessler *et al.*, 1971).

This kind of evaluation enabled us to determine the number of breaks per DNA molecule for the complete yeast genomic DNA and for individual yeast chromosomes. However, only the larger chromosomes corresponding to bands 6–13 could be analysed by evaluation of the photonegatives of ethidium bromide-stained gels, since the smaller chromosomes of irradiated cells are superimposed by the smear of low molecular-weight DNA in the gel.

Chemicals

Low melting-point (Sea Plaque) and LE agarose were from FMC (Rockland, ME), and S1 nuclease and restriction enzymes from Boehringer Mannheim (Mannheim, FRG). Zymolyase was obtained from Miles (Indianapolis, IN), proteinase K from Serva (Heidelberg, FRG) and phenylmethylsulphonylfluoride (PMSF) from Sigma (St Louis, MO). Nitrocellulose membranes were from Schleicher and Schuell (Keene, NH).

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