Analytical Biochemistry 443 (2013) 269–281

Contents lists available at SciVerse ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Trapping and breaking of in vivo nicked DNA during pulsed field gel electrophoresis

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ARTICLE INFO

Article history:
Received 1 August 2012
Received in revised form 30 May 2013
Accepted 4 June 2013
Available online 14 June 2013

Keywords:
Field strength
Time of electrophoresis
DNA trapping
Single-strand breaks
Double-strand breaks

ABSTRACT

Pulsed field gel electrophoresis (PFGE) offers a high-resolution approach to quantify chromosomal fragmentation in bacteria, measured as percentage of chromosomal DNA entering the gel. The degree of separation in pulsed field gel (PFG) depends on the size of DNA as well as various conditions of electrophoresis such as electric field strength, time of electrophoresis, switch time, and buffer composition. Here we describe a new parameter, the structural integrity of the sample DNA itself, that influences its migration through PFGs. We show that subchromosomal fragments containing both spontaneous and DNA damage-induced nicks are prone to breakage during PFGE. Such breakage at single-strand interruptions results in artifactual decrease in molecular weight of linear DNA making accurate determination of the number of double-strand breaks difficult. Although breakage of nicked subchromosomal fragments is field strength independent, some high-molecular-weight subchromosomal fragments are also trapped within wells under the standard PFGE conditions. This trapping can be minimized by lowering the field strength and increasing the time of electrophoresis. We discuss how breakage of nicked DNA may be mechanistically linked to trapping. Our results suggest how to optimize conditions for PFGE when quantifying chromosomal fragmentation induced by DNA damage.

Chromosomal lesions lead to cell cycle arrest in eukaryotes and block progression of replication forks in prokaryotes, posing serious problems for the accurate and timely duplication of the genome and its physical integrity [1–3]. Although all DNA lesions, including single-stranded nicks (SSNs)[1] and single-stranded gaps (SSGs), are harmful, unrepaired double-strand breaks (DSBs) are the most deleterious ones and can cause genome loss and cell death [4–7]. Studies on the mechanisms of chromosomal fragmentation are of critical importance for two reasons. First, all known DNA-damaging agents, both naturally occurring and human-made, induce chromosomal fragmentation in replicating cells [8–10]. Second, cells that cannot repair fragmented chromosomes die, whereas those that can experience genome instability.

Quantification of chromosomal fragmentation relies on physical methods with a long linear range of detection, and pulsed field gel electrophoresis (PFGE) has proven to be the best technique for this purpose. Conceived and developed during the 1980s, PFGE fractionates chromosome-size DNA molecules [11,12] (for reviews, see Refs. [13–15]) and has been used extensively in the areas of genome characterization [16–18] (reviewed in Refs. [19,20]) and epidemiological studies [21,22] (reviewed in Refs. [23,24]). In PFGE, the DNA is subjected to alternating electric fields with a constant reorientation angle over a long period of time, and the migration of DNA is controlled by a variety of factors, including field strength, pulse time, gel concentration and buffer composition, and temperature and time of electrophoresis [25,26]. A peculiar characteristic of PFGE is that even 30-kbp relaxed circular DNA molecules cannot enter the gel [27–30], whereas linear fragments of up to 10 Mbp do [31–33]. This property makes PFGE an ideal technique for chromosomal fragmentation studies in bacteria, where the linearized chromosomes and chromosomal fragments are efficiently separated from the (unbroken) circular chromosome because the former enter the gel, whereas the latter stays within the wells [5,6,34–37].

The use of PFGE to quantify chromosomal fragmentation in bacteria was initiated in 1997 by Michel and coworkers [37], who based their approach on an earlier demonstration in yeast that (i) a circular 305-kbp chromosome does not enter the gel and (ii) it does enter the gel when it is linearized [33]. The technique has since been successfully used in a number of chromosomal fragmentation studies [5,6,34,35,38–40]. However, with the exception of the high energy ionizing radiation (gamma rays and X-rays),

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[1] *Abbreviations used:* SSN, single-stranded nick; SSG, single-stranded gap; DSB, double-strand break; PFGE, pulsed field gel electrophoresis; UV, ultraviolet light; HMW, high-molecular-weight; EDTA, ethylenediaminetetraacetic acid; TBE, Tris–borate–EDTA; PFG, pulsed field gel; LLFS, long run at low field strength; SHFS, short run at high field strength; DBA, different batch of agarose; LMW, low-molecular-weight.

0003-2697/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ab.2013.06.001

DNA-damaging conditions rarely generate direct DSBs. At the same time, no one has ever asked whether single-strand interruptions in duplex DNA, generated in vivo either in the course of repair of DNA damage or due to DNA metabolism defects, could influence its electrophoretic behavior during PFGE.

Recently, while studying the mechanisms of ultraviolet light (UV)-induced chromosomal fragmentation in *polA* and *ligA* mutants of *Escherichia coli* that are defective in joining of Okazaki fragments in newly replicated DNA, we suspected that long DNA molecules were broken at the nicks due to high electric field strength used during standard PFGE conditions. While testing this idea, we found that nicked linear DNA indeed was further fragmented during PFGE. However, contrary to our expectations, reducing field strength did not reduce the conversion of nicks into breaks, demonstrating that this phenomenon was field strength independent. Although we detected field strength-independent breakage of high-molecular-weight (HMW) DNA at nicks in a variety of experiments, including assays with defined subchromosomal fragments, we found that one particular batch of agarose caused especially prominent nick-dependent trapping of large chromosomal fragments within wells. These nicked HMW species could be forced into the gel by lowering the field strength and increasing the time of electrophoresis, but once these nicked species entered the gel, they were further broken, likely at preexisting nicks, mechanistically linking breakage at nicks with trapping inside the gel. Our findings demonstrate that care should be taken when interpreting molecular weight distribution of the chromosomal fragmentation results if the DNA is expected to contain nicks.

Materials and methods

Bacterial strains, growth conditions, and chemical reagents

All *E. coli* strains used in this study are K-12 derivatives and are described in Table 1. All strains were grown in LB (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter of broth, pH to 7.4, with 250 μl of 4 M NaOH; LB agar contained 15 g of agar/L LB broth) at 28 °C unless stated otherwise. When required, antibiotics were added to the following final concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; tetracycline, 10 μg/ml. DNA-damaging agents were used at the following final concentrations: AZT, 500 ng/ml; phleomycin, 2.5 or 10 μg/ml; nalidixic acid, 25 μg/ml; fluorouracil, 100 μg/ml; hydroxyurea, 100 mM. Agarose used in this study was obtained from Denville Scientific, Fisher, and Gibco, whereas enzymes used in various assays were purchased from New England Biolabs.

Chromosomal fragmentation

Labeling of the chromosomes of growing cultures with ^{32}P, as well as UV treatments, was done exactly as described by Khan and Kuzminov [5]. Briefly, cells were grown aerobically at 28 °C in the presence of 2 to 4 μCi/ml [^{32}P]orthophosphoric acid (MP Biomedicals) until they reached $A_{600} \sim 0.3$. At this point, DNA-damaging agents were added directly to the cultures, which were then transferred to a 37 °C shaker for 2 h. For UV assays, the cultures were harvested and the cell pellets were suspended in volumes of sterile 1% NaCl containing 0.01% Triton X-100 (Sigma) to yield an absorbance of approximately 0.6. The UV irradiation was performed at 28 °C in a dark room lit by yellow lamps (F15T8-GO, General Electric) to avoid photoreactivation. Following irradiation, the cultures were diluted 1:1 with a sterile no-salt 2× LB solution and transferred to a 37 °C shaker for either 10 min or 2 h. For spontaneous chromosomal fragmentation studies, the strains were labeled, harvested, and concentrated in Triton X-100 and NaCl as described above for the UV assay. After normalizing the suspensions to absorbance of approximately 0.6, the cultures were diluted 1:1 with 2× LB (without NaCl) and transferred to a 37 °C shaker for 2 h.

Agarose plugs and PFGE

After completion of the required incubations, cultures from a volume of 0.5 ml were harvested for each plug. The cell pellets were washed once in 1 ml of sterile TE buffer (10 mm Tris–HCl and 1 mm ethylenediaminetetraacetic acid [EDTA], pH 8.0) and resuspended in 60 μl of TE buffer. To make agarose plugs, 5 μl of 5 mg/ml proteinase K solution (Roche Applied Science; final concentration in plugs 200 μg/ml) and 65 μl of molten agarose in lysis buffer (1.2% agarose in 1% lauroylsarcosine, 50 mm Tris–HCl, and 25 mm EDTA, pH 8.0) were mixed with the cells, and the suspensions were transferred to plug molds (Bio-Rad). The solidified agarose plugs were submerged in 1 ml of the lysis buffer (1% lauroylsarcosine, 50 mm Tris–HCl, and 25 mm EDTA, pH 8.0) and incubated overnight at 60 °C. After completion of digestion, the lysis buffer was replaced with TE buffer and the plugs were stored at 4 °C until used. Unless specifically mentioned, ½ plugs were used for electrophoresis.

In some assays where plugs were to be restriction digested, a modified method of plug lysis was used. In this procedure, cell pellets were washed and suspended in 65 μl of TE buffer, mixed with 65 μl of molten plain agarose (1.2% agarose in water), and transferred into plug molds. Once solidified, these plugs were shifted to glass tubes containing 1 ml of 1 mg/ml lysozyme in TE buffer and incubated at 37 °C for 1 h. Following this, the plugs were removed from TE buffer and transferred to fresh tubes containing 1 ml of 1 mg/ml proteinase K in lysis buffer (1% lauroylsarcosine, 50 mm Tris–HCl, and 25 mm EDTA, pH 8.0). The tubes were shifted to 60 °C, and plug lysis was continued for 15 to 18 h.

All electrophoresis runs were performed on 1% agarose gels in 0.5× Tris–borate–EDTA (TBE) buffer at 12 °C in a Bio-Rad CHEF-DR II PFGE system operating with initial and final switch times of 60 and 120 s, respectively. Field strengths between 1 and 6 V/cm and time of electrophoresis between 10 and 150 h are specified in descriptions of individual experiments. In most of the gels, yeast

Table 1
Strains used in this study

Strain	Relevant genotype	Source or reference	Properties
AB1157	wild-type strain	[58]	Repair proficient
SK129	*recB270*(Ts) *recC271*(Ts)	[59]	Defective in DSB repair and DNA degradation
AK107	SK129 *dut-1 zic4901*::Tn*10*	[38]	Defective in DSB repair and also incorporates uracil in DNA
AK25	AB1157 *polA12*(Ts)::Tn*10*	Lab collection	Defective in excision DNA repair
GR501	GR523 *ligA251*(Ts)	[60]	Defective in sealing DNA nicks
LA20	GR501 *ypeB*::*kan*	[61]	Defective in sealing DNA nicks
SRK312	SK129 *polA12*(Ts)::Tn*10*	SK129 × P1 AK25	Defective in DSB repair and excision repair
SRK322	SK129 *ligA251*(Ts)	SK129 × P1 LA20	Defective in DSB repair and sealing DNA nicks
SRK301	SK129 *ΔuvrA277::Tn10*	[5]	Defective in DSB repair and nucleotide excision repair