THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

A study of DNA discontinuities in Sacchromyces cerevisiae cells

by Éva Hegedüs

Supervisor: Prof. Dr.Gábor Szabó

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR CELL AND IMMUNBIOLOGY

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1. Introduction

There is relatively a large body of data and knowledge about the lower levels of chromatin hierarchy, but information related to the higher order chromatin organization is less abundant and often inconsistent. Our group got interested in the features of chromatin loops representing one of the higher levels of chromatin organization through phenomena of DNA fragmentation occurring in this size range. DNA from viable, non-apoptotic eukaryotic cells exhibits ds fragmentation focused around ~50 kb, which is similar to the average size of functional units of chromatin (replication and transcription units). Sequences involved in pathological gene rearrangements are overrepresented in the breakpoints of loop-size DNA fragmentation both in healthy and apoptotic cells, suggesting that these regions can be hot-spots for translocations.

1. 1. Aims

A. I wished to answer a number of basic questions concerning the mechanisms and biological significance of loop-size chromatin fragmentation, making use of the possibilities offered by the yeast cell systems.

   My specific aims were to determine

   – the correlation of discontinuities on the two strand of DNA

   – to examine the cell cycle-dependence of loop-size chromatin fragmentation

   – to compare the patterns of fragmentation in individual chromosomes

   – to examine the possibility that chromatin fragmentation might exhibit superhelicity-dependence

   – to examine chromatin fragmentation in *Schizosaccharomyces pombe* cells

   – to characterize the true size distribution of fragments

   – to examine the behaviour of the ribosomal DNA cluster (rDNA locus, composed of the many units comprising identical sequences) in loop-sized fragmentation

   – to map nicks and RNA/DNA hybrids in rDNA copies

B. My aims included development of novel methods for screening discontinuities supposed to be predilection points for translocations.

– development of flow cytometric methods for detection of gene rearrangements or single-strand discontinuities
– development of new gel electrophoretic technic for detection and mapping of gene rearrangements or single-stranded discontinuities

2. Background

2. 1. The eukaryotic chromatin structure

Eukaryotic chromatin appears to be organized into supercoiled loops anchored to the putative nuclear matrix/ scaffold with a periodicity of 70-220 kb at S/MAR (Scaffold / Matrix Attachment Region) sequences of 600 – 3000 bp in length. S/MARs have no unique consensus sequences, but often form alternative secondary structures including base-unpairing regions that become single-stranded upon superhelcial stress. The loop arrangement appears to be highly dynamic, also in the sense that the cells most likely do not use allthe potential attachment points at the same time. The role of loops in gene regulation is unknown. Central to our present concept of such regulation are combinations of specific posttranslational histone modifications that constitute the "epigenetic" level of regulation with significant differences between mammalian and yeast cells.

Comparison of human and yeast genomes:

	Genome size(Mb)	**Chr. (1n) number**	**Chr. size**	**Gene number**	**Repeat %**	**Average transcript / gene size (kb)**	**GC content**
H. sapiens	3300	23	50-263 Mb	30000	46	25	41.5 %
S. cerevisiae	12 +rDNA	16	230-2200 Kb	5700	2.4	2	38.3 %
S. pombe	13.8	3	3.5-5.7 Mb	4900	0.35	1.4	36 %

The most significant repeats are those of the ribosomal DNA (rDNA) in yeast cells:

	rDNS copy number	**rDNS copy length (kb)**	**rDNS localization**
S. cerevisiae	100 -200	9.1	Chr. XII.
S. pombe	100 -200	10.4	Chr. III.

2. 2. Features of the structural hierarchy of chromatin is reflected by the DNA fragmentation phenomena

The phenomenon of nucleosomal or 50–300 kb DNA fragmentation was extensively described in apoptosis. The main symptoms of apoptosis were also described in the case of yeast cells.

~50 kb fragmentation of DNA can also be observed when chromatin, nuclei or living cells of normal, non-apoptotic human or mouse cell lines are treated with protein denaturing agents, or directly lysed by alkali or (in the case of nuclei and chromatin) treated with DNase I applied at low concentrations.

S1 nuclease treatment of agarose embedded, deproteinized chromatins of mammal and yeast cells (to avoid the mechanical shearing) also cause DNA fragmentations. The digestibility with S1 nuclease could mark locally denatured regions or single-stranded (ss) discontinuities in the DNA. Using the method of urea-agarose denaturing gel electrophoresis, true ss discontinuities were revealed.

Using field inversion single cell gel electrophoresis performed in alkaline conditions our group has shown that the chromatin of non apoptotic human and budding yeast cells becomes disintegrated to granules containing ~48 kb DNA.

RNase H sensitivity and labelling by an anti-RNA/DNA hybrid monoclonal antibody revealed RNA/DNA hybrids near ss discontinuities, nicks, in nuclear halo samples.

After cloning and sequence analyses, the fragment ends were also found to contain many repetitive elements as well as a consensus sequence that was also found in the MLL bcr (breakpoint cluster region), known to be involved in in loop-size, apoptotic DNA fragmentation.

2. 3. Transient discontinuities in the genomic DNA upon physiological activity of cells

Transient discontinuities, which can also be sources of genome instability, often occur during the cell cycle. These discontinuities can be present on one (nick, gap) or on both strands of the DNA double helix and may be related to replication, transcription, DNA repair or recombination. Selected examples are mentioned below.

The relaxation of torsional stress upon replication and transcription is carried out by topoisomerase enzymes cutting one or both strands. After replication, nicks and gaps can remain in the DNA, which can cause ds breakage in the next cell cycle.

The transcription-induced negative-superhelicity can promote the formation of R-loops (RNA/DNA hybrids), upon annealing of the nascent RNA to its DNA template. The topoisomerase I indirectly blocks the formation of R-loops, because it relaxes the negative supercoils, and binds to and activates the ASF/SF2 (alternative splicing factor / splicing factor 2) directly, promoting the maturation of mRNA. Interestingly, in the absence of ASF/SF2, ds breakage and high molecular weight DNA fragments (~50 kb) appear.

3. Materials and methods

3. 1. 1. *Preparation of agarose-plugs containing yeast chromosomes*

S. cerevisiae cells (WDHY 199 strain;MATa, leu2-3,112 trp1-289 ura3-52 his7-2 lys1-1) were grown under nonselective conditions in liquid YPD medium (1 % yeast extract, 2 % bactopepton, 2 % glucose, pH 5) at 30°C, to a concentration of ~2×10^7 cells/ml (logarithmic phase, OD_{600}=1) or to ~4×10^9 cells/ml (stationary phase, OD_{600}=3.5). Cells were washed twice in 50 mM EDTA (pH 8), then the cells were resuspended in digestion solution (0.9 M sorbit / 0.125 M EDTA / 100 mM dithiothreitol (DTT)) containing 2 mg/ml Lyticase enzyme (Fluka). Samples were mixed with an equal volume of 1 % low melting point (LMP) agarose (Sigma Chemical Company) dissolved in 0.9 M sorbitol / 0.125 M EDTA. Aliquots were allowed to harden in sample molds at 4°C for 5 minutes, then placed into 0.9 M sorbitol / 0.125 M EDTA at 37°C for 6 hours. Each plug contained ~3×10^8 cells.

The S. pombe cells (L972h^- strain; wild type) were grown in liquid YEL (0.5 % yeast extract, 3 % glucose, pH 5) at 30°C, to a concentration of ~5×10^6 cells/ml (log phase) or to ~2×10^8 cells/ml (stationary phase). Cells were washed twice in 50 mM EDTA (pH 8), then the cells were resuspended in CPES buffer (25 mM citric acid, 0.12 M Na_2HPO_4, 20 mM EDTA, 1.2 M sorbitol) containing 15 mg/ml Lysing enzyme (Sigma). Samples were mixed with an equal volume 1% LMP agarose dissolved in 0.9 M sorbitol / 0.125 M EDTA. Aliquots were allowed to harden in sample molds at 4°C for 5 minutes. Each plug contained ~1.5×10^7 cells.

The plugs containing yeast protoplast were digested in lysing solution (0.5 mg/ml Proteinase K, 0.5 M EDTA, 10 mM Tris/HCl, 1% SDS, pH 8) at 54°C for 2 days, then washed with TE (10 mM Tris/HCl, 2 mM EDTA, pH 8) and stored in TE at 4°C.

3. 1. 2. Synchronization of the S. cerevisiae cells

Synchronization in G_1 phase: S. cerevisiae cells were grown to OD_{600}=1, then α-factor (Sigma) was added into the medium to 20 μg/ml (final concentration). *Synchronization in S phase:* S. cerevisiae cells were grown to OD_{600}=1, then 200 mM hydroxyurea (Sigma) was added into the medium and incubated for 1.5 hours. *Synchronization in* G_2 / M *phase:* S. cerevisiae cells were grown to OD_{600}=1, then nocodazole (Sigma) was added into the medium to 20 μg/ml (final concentration) and incubated for 1.5 hours. The results of synchronizations were controlled by flow cytometry.

3. 1. 3. Assessment of the viability of S. cerevisiae cultures

The colony forming assay was used to determine the viability of yeast cultures. 100-100 cells from the logarithmic or stationary cultures were applied on YPDA plates, (YPD medium, 2 % agar) by streaking. The plates were incubated at 30°C for 3 days and colonies were counted. Viability of the cultures was scored based on the average of the colony numbers counted on five parallel plates.

3. 1. 4. Enzyme treatment of agarose plugs

Agarose plugs were treated with 0.75 mM phenyl-methyl-sulfanyl-fluoride (PMSF) at 37°C, for 10 minutes, in order to inactivate any residual protease activity and then washed three times with TE.

S1 nuclease digestion: Plugs were preincubated in S1 buffer (0.2 M NaCl, 50 mM Na-acetate, 1 mM $ZnSO_4$, 0.5 % glycerol) for 3×20 minutes and then incubated with 500 U/ml S1 nuclease at 37°C, for 1.5 hours.

DNA polymerase I treatment: Plugs were preincubated in 1×polymerase buffer (50 mM Tris-HCl (pH 7,5), 10 mM $MgCl_2$, 1 mM DTT) for 3×20 minutes and incubated with 150 U/ml DNA polymerase I (Fermentas Life Sciences Inc.) in 1×polymerase buffer supplemented with 2,5 μM dATP / dCTP / ddGTP / biotin-dUTP or 2,5 μM dATP / dGTP /

ddCTP / biotin-dUTP or 2,5 μM dCTP / dGTP / ddATP / biotin-dUTP, at 37°C-on for 1.5 hours.

Sfi I digestion: Plugs were preincubated in 1× G buffer (10mM Tris-HCl (pH 7,5),10 mM $MgCl_2$, 50 mM NaCl, 0,1 mg/ml BSA) and then incubated with 150 U/ml Sfi I restriction endonuclase (Fermentas) for 16 hours at 50°C.

Sma I digestion: Plugs were preincubated in 1× Tango buffer (33mM Tris-acetát (pH 7,9),10 mM Mg-acetát, 66 mM K-acetát, 0,1 mg/ml BSA) and then incubated with 150 U/ml Sma I restriction endonuclase (Fermentas)) for 16 hours at 30°C.

Pvu II digestion: Plugs were preincubated in 1× G buffer and then incubated with 150 U/ml Pvu II restriction endonuclase (Fermentas) for 16 hours at 37°C.

3. 1. 5. Pulsed-field gel electrophoresis: FIGE, CHEF

Field inversion gelelectrophoresis (FIGE) was carried out at 5 V/cm, in 1×TAE, applying an MJ Research PPI 200 Power Inverter, in a cold room. Running parameters were set to maximize resolution in the 50-400 kb range (Program 5) or upto 2000 kb (Program 9).

Neutral FIGE: 1 % agarose gels were prepared in 1×TAE (40 mM Tris-acetate, 1 mM EDTA). After gelelectrophoresis, gels were stained with 0.5 μg/ml ethidium bromide (EBr).

Urea/heat-denaturing FIGE: Agarose plugs were equilibrated in 1×TAE containing 8 M urea for 1 hours. Heat denaturation was performed at 80°C for 5 minutes. 1×TAE containing 1 M urea was used as the electrophoresis buffer. To prepare the urea-agarose gels, urea was added (at 1 M final concentration) to the 1 % agarose solution prepared in 1×TAE. After electrophoresis, gels were washed in 1×TAE for 2 hours, then in 100 mM NaCl for 2 hours, to renature DNA. Gels were stained with 0.5 μg/ml EBr for 30 minutes.

CHEF-DR II PFGE system was used for the separation of chromosomal DNA molecules.The gelelectrophoresis was carried out in 1 % agarose gel in 0,5×TBE buffer (45 mM Tris-borát, 1 mM EDTA, pH 8,3) at 14°C.

3. 1. 6. Two-dimensional gel electrophoresis

The chromosomal DNA molecules were sepateted from each other by CHEF-DR II PFGE system, in a first dimension. Separation of the loop-size fragments derived from the

chromosomes after treatment with S_1 nuclease or urea – heat denaturation was carried out by FIGE run in a second dimension.

3. 1. 7. Southern-blot

The standard and urea/heat-denaturing agarose gels were transferred to Hybond-N^+ nitrocellulose membranes (Amersham Pharmacia Biotech) using a BIO-RAD vacuum blotter. The membranes were dried for 30 min at 80 ºC and UV cross-linked ($1.2x10^5$ μJ/cm^2). The blotted, denatured DNA was prehybridized for 3 hours at 55 ºC in 30 ml prehybridization solution (1 m/v % BSA, 0.5 M Na_2HPO_4, 7 m/v % SDS, 1 mM EDTA, 10 μg/ml salmon sperm DNA), and was hybridized for 15 hours with single-strand-specific probes for the rDNA gene cluster. The ds PCR product of 1405 bp length was used as template DNA for subsequent probe preparation. Labelling with ^{32}P was performed either by random primer labelling (ds probe; using an RediPrime Kit, Amersham) or "linear amplification" (using a single primer) to prepare strand-specific probes. In these reactions 2.5 U Taq polymerase (Fermentas Life Science, Maryland, USA) was used, in 50 μl of 1×reaction buffer (10 mM Tris-HCl, 50 mM KCl, 0.08 % Nonidet P-40, pH 8.8) supplemented with 3 mM $MgCl_2$, containing 50 ng template DNA, 20 pmol of primer, and the nucleoside-triphosphates. dATP, dTTP and dGTP were used at 0.25 mM, dCTP at 5 μM concentration (all from Promega Life Science, Madison, USA), and for each labelling reaction 5μl [$\alpha^{32}P$]-dCTP (6000 Ci/mmol, 10 mCi/ml; Institute of Isotopes LTD, Budapest) was added. In the first reaction cycle, denaturation was at 94°C for 3 min, annealing at 60°C for 1.5 min, polymerization at 72°C for 1.5 min; this was followed by 45 cycles when denaturation was at 94 °C for 1.5 min, annealing at 60°C for 50 sec, polymerization at 72°C for 1.5 min. The probes were purified on Sephadex G-25. After hybridization, the membranes were washed three times at 60 ºC with a washing solution (40 mM Na_2HPO_4, 1 m/v% SDS, 1 mM EDTA). The signal was detected by Phospho-screen (Kodak) and visualized by a BIO-RAD Phospho-Imager.

3. 1. 8. „Ab-Southern"

Plugs containing chromatin of S. cerevisiae were used to incorporate biotin-dUTP into the DNA by nick translation, then these samples were digested with Pvu II restriction endonuclease. Plugs treated with or without urea/heat-denaturation were run on urea-agarose gels, then transferred to Hybond-N^+ nitrocellulose membranes. Membranes were

prehybridized for 1 hour at room temperature in 5 ml prehybridization solution (5 % milk powder, 0,2 % Tween-20 / PBS), then labeled by mouse anti-biotin primary antibody used at a dilution of 1:1000 in 5 ml hybridization solution for 16 hours at 4°C. After washing with 0,2 % Tween-20 / PBS for 5×5 minutes, the membranes were incubated with goat anti-mouse IgG antibody conjugated with horseradish peroxidase (dilution 1:2000) for 1.5 hours at room temperature. The signal was detected by chemiluminescence. (This novel method has been worked out and named by myself.)

3. 2. 1. Detection of ss DNA regions and nicks by flow cytometry

Enzymatic cleavage at single-stranded regions by S_1 nuclease. 100 ng biotin/6FAM labeled PCR product was digested by 0.00001 – 1 U S_1 nuclease in 50 µl 1× S_1 buffer at 20°C for 40 min., in the dark. The reactions were stopped by the addition of 125 µl of 0.1 M EDTA. DNA was precipitated for 2 hours after the addition of 125 µl of 0.3 M Na-acetate and 750 µl –20°C abs. ethanol. After centrifugation, DNA was dissolved in 50 µl phosphate buffered saline (PBS, pH 7.4).

Chemical cleavage at single-stranded regions by hydroxylamine / piperidine treatment. 100 ng biotin/6FAM labeled PCR product dissolved in 2 µl TE was added to different amounts of hydroxylamine, in a final volume of 100 µl set by PBS (pH 6.0), and treated at 37°C for 2 hours in the dark. The 0.5 M hydroxylamine stock solution was prepared in 2 M tetraethylammonium chloride (pH 6.0, adjusted by dietilamin). Reaction was stopped by the addition of 125 µl of 0.1 M EDTA. DNA was precipitated, dissolved in 50 µl sterile dH_2O, and mixed with an equal volume of 1 % piperidine, and incubated at 90°C for 30 min. in the dark. Finally, DNA was precipitated and dissolved in 50 µl of PBS.

Generation and detection of nicks. Biotin/6FAM labeled PCR products containing a single nick were prepared and treated with 10 U of E. coli DNA polymerase I, in 25 µl of 1×polymerase buffer containing dATP, dCTP, dGTP each at 40 µM, dTTP at 2 µM and fuorescein–12–dUTP at 40 µM final concentration, at 16°C for 2 hours.

Binding of PCR products to microbeads, flow cytometric analysis. 10000 streptavidin coated polymeric microbeads (Polyscience Inc.; 6 µm diameter) were added to 50 µl of the biotin/6FAM labeled PCR products in PBS and incubated at room temperature for 40 min in the dark. The microbeads were washed twice in 500 µl of PBS, then resuspended in 500 µl of PBS. The microbeads carrying fluorescent dye labeled PCR products were

measured by a Becton-Dickinson FACScan flow cytometer. Fluorescence signals in the FL1 and FL2 channels were detected through the 530/30 and the 585/42 interference filter of the instrument, respectively. The applied laser power was 15 mW. The data collected were analyzed by BDIS Cell Quest 3.3 software (Becton-Dickinson).

4. Results

4. 1. Loop-size DNA fragmentation in yeast cells

4. 1. 1. Detection of discontinuities in yeast genomic DNA by enzimatic and denaturing treatments

~100 % of the cells derived from logarithmic or stationary cultures yielded healthy, growing colonies examined by colony-forming assay, clearly showing that normal, non-apoptotic cells were used throughout my experiments.

Agarose embedded deproteinized S. cerevisiae and S. pombe chromatin exposed to S1 nuclease digestion, or upon urea/heat denaturation, yielded DNA fragments of ~50 kb, due to the presence of single-strand (ss) discontinuities (nicks), on pulsed-field gels.

The comparison of S1-treated and non-treated samples on urea-denaturing gels resulted in similar patterns of fragmentation, demonstrating that the nicks are close to each-other on the two strands of the DNA.

4. 1. 3. Cell cycle-dependence of loop-size fragmentation

I have synchronised the S. cerevisiae cells in G1-, S- or G2/M- phases. Agarose plugs from different cell-cycle phases gave identical patterns of fragments after S1 nuclease treatment or urea/heat denaturation, demonstrating that there is no conspicuous cell-cycle-dependence of the phenomenon.

Agarose plugs from logarithmic and stationery S. pombe cells also gave identical patterns after S1 nuclease treatment or urea/heat-denaturation, similarly to S. cerevisiae.

4. 1. 4. Lack of superhelicity-dependence of loop-size fragmentation

One nick per loop could also occur in a nonspecific manner if the initial cleavage is by a superhelicity-dependent nucleases. In this case the appearance of the first nick in a loop would abolish the superhelicity so the appearance of another nick is unlikely in the same loop, leading to one nick per superhelical loop To examine this alternative possibility, I have incubated the agarose plugs containing S. cerevisiae spheroplasts in isotonic solutions supplemented with different concentrations of EBr. It is known that the negative superhelical DNA first becomes relaxed, then gets overwound at increasing concentrations of the intercalating EBr. I have demonstrated that the presence of different concentrations of EBr, i.e. different superhelical status of DNA, does not affect the appearance of loop-size fragmentations observed either after S1 nuclease treatment or urea/heat denaturation. These results exclude the the alternative mechanism mentioned above and lend further support to the hypothesis that the discontinuities detected are specific sites delimiting, perhaps anchoring, the chromatin loops.

4. 1. 5. Treatment of individual chromosomes by S1 nuclease or urea/heat denaturation

The 2D-gel electrophoretic analyses of individual chromosomes resulted in loop-sized fragmentation focused at ~50 kb, after either S1 nuclease treatment or urea/heat denaturation, excluding various genetic and epigenetic factors from the decisive factors of phenomenonin view of the differences among the chromosomes and species.

1. 1. 6. Breakpoints on S. cerevisiae chromosome I.

Using CHEF, I have determined the true size distribution of fragments that appeared to be focussed at ~50 kb in FIGE. In line with the expectation for chromatin loops, this size range spanned an interval of 20-200 kb using a CHEF program which resolves the fragments upto 400 kb.

The distribution of nicks in S. cerevisiae chromosome I was studied by Southern-blotting. The S1-sensitive sites appeared to be distributed somewhat randomly but with the incidence of nicks focused at certain regions. These regions are equally revealed when S1 digestion precedes or follows restriction digestion with Sfi I or Not I. At the resolution and sensitivity achieved in my mapping of S1-sensitive sites along chromosome I, the patterns of

fragmentation appear to reflect a partially stochastic, in part sequence/site specific definition of these sites. The localisation of nicks is correlated with ARS sequences. This fact can suggest that nicks have functional significance in processes of replication.

4. 1. 7. Mapping of nicks in the S. cerevisiae rDNA locus

The ~1.5 Mb rDNA cluster desintegrated into 20 – 200 kb fragments as shown with rDNA specific Southern probes after S1 nuclease treatment or urea/heat denaturation, similarly to the behavior of the total DNA. This incidence implies that every 2-20. rDNA unit should contain a nick (every 11. unit on the average).

The rDNA cluster was cleaved to its units by restriction endonucleases cutting only once in each rDNA unit. The distribution of the nicks was studied on the local scale by Southern blot mapping of the S1 sensitive sites in rDNA copies. The nicks appeared to be focused at particular regions.

To map these sites, the rDNA units were separated into differentially migrating complementary strands upon urea/heat-denaturation and agarose gel electrophoresis. The single-stranded (ss) DNA fragments run differently and much faster than the ds DNA they are derived from. I have shown that the differential electrophoretic migration of the two strands of the same size is due to their different base composition leading to different overall conformational characteristics even in the presence of the denaturing agent.Comparison of the sequence composition of the opposing strands for all the DNA fragments analyzed revealed that their separation depends both on the C/G and (A+T)/(C+G) ratios.

The localisation of nicks on the two strands of rDNA was mapped by Southern hybridization using ss-specific probes. Nicks manifested after denaturation were also focused at particular regions of rDNA units.

A nick was detected near the replication fork barrier (RFB) of the rDNA units. Three other nicks were shown to be likely artefacts of restriction endonuclese digestion.

4. 1. 8. Mapping of nicks by Ab-Southern

Biotin-dUTP incorporation was not detected in Pvu II fragments carrying the ARS sequences of rDNA examined by „Ab-Southern”. Thus, I have demonstrated that nicks are not present in the close proximity of the ARS sequences of rDNA. On the other hand, the presence of nicks could be detected in an RFB containing fragment by this approach. These

data suggest that the relative co-localization of the nicks with ARS sites observed in chromosome I may reflect an overall spatial relationship rather than molecular proximity.

4. 2. Development of methods for the detection of DNA discontinuities and other genetic defects

To test our strategy for the detection of deletions / insertions in a DNA molecule, we prepared heteroduplices between a 6FAM / biotin labeled 725 bp and a Cy3 / biotin labeled 541 bp PCR product, both being amplified from the same region of the plasmid containing the MLL bcr using identical antisense primers.

After reannealing the denatured molecules, the combinations obtained included two types of hybrids. These products were then cleaved at the single-stranded regions by S1 nuclease or hydroxylamine-piperidine, then bound to streptavidin coated microbeads and measured by flow cytometry. The heteroduplices with overhangs were much more susceptible to the enzyme than the homoduplices.

To test if single-strand discontinuities can be detected using nick-translation in conjunction with microbead analysis, a 725 bp long region of the MLL bcr amplified using unlabeled sense and antisense primers was cleaved by the Xba I restriction enzyme, yielding a single cut in the sequence, and the cleaved DNA was hybridized with an uncleaved, biotinylated PCR product at a ratio of 1:1. After annealing, the combinations obtained included the two original amplicons as well as two hybrids containing a nick. These nicks could be translated by E. coli DNA polymerase I holoenzyme incorporating fluorescein–12–dUTP into the newly synthesized DNA strands. The fluorescence of the biotin labeled hybrids immobilized on streptavidin coated microbeads was determined by flow cytometry.

We have elaborated a urea-agarose gelelectrophoretic based technique for the analyses of the two separated strands of the urea/heat-denatured DNA, applicable in many different scenarios including detection of strand specific nicks.

5. Discussion

5. 1. Loop-size chromatin fragmentation in yeast cells

The ss discontinuities detected in agarose embedded DNA derived from S. cerevisiae or S. pombe cells of ~100% viability might have arisen in the moment of the lysis of the cells or, more likely they must have been present in the genomic DNA before cell lysis, masked by proteins and/or RNA molecules, in line with earlier observations in our group.

The size of the fragments was not influenced by the superhelicity of chromatin loops ruling out the interpretation that relaxation of DNA by a first nick in a superhelical loop arrests the accumulations of additional nicks in a same loop giving rise to the formation of one nick per a loop in average.

Despite particular differences among *S. cerevisiae* and *S. pombe* chromosomes, they are rather uniform in terms of gene density, sequence compositions as well as epigenetic characteristics including the degree of packaging/compaction. The fact that marked differences in the incidence of DNA breaks could not be revealed between the different chromosomes suggests that the phenomenon is related to general characteristics of the chromosomes rather than to specific genetic or epigenetic marks. A typical *S. cerevisiae* chromosome contains mainly unique, non-repetitive sequences except their telomeric regions. However, more than half of chromosome XII. is composed of tandem repeats of rDNA. The behavior of this chromosome was not different from the others. Interestingly, every 11^{th} transcription unit of identical sequence composition was shown to contain a specific nick, localized at the RFB, suggesting that the factors determining the site of these ss discontinuities are of epigenetic nature.

I have demonstrated also by a new method I name „Ab-Southern" that this nick maps to the region of RFB. The NTS2 subregion of the rDNA transcription units contains the RFB sequences. The Fob 1 protein is known to bind to this sequence and in this manner stops the progress of replicating fork in one direction. Fob1 and replication, as well as topoismorease I dependent DNA breaks were indeed detected in these sequences.

5. 2. Development of methods

I have developed a new method to analyse the two complementery strands of DNA in the range of 1-20 kb. The method is highly reproducible and it is applicable in various scenarios when single-strand discontinuities are to be mapped.

I have developed flow cytometric assays for detection of various genetic alterations using PCR products immobilized on microbeads. Deletions and insertions are frequent genetic alterations involved in cancer and genetic diseases as commonly known, and also contribute to normal mutation rates. Insertional mutagenesis involving mobile genetic elements and viruses, unrepaired 1 nucleotide slippage of replication, triplet expansion are among the most frequent causes of deletions and insertions and these are detectible by approaches based on flow-cytometric heteroduplex analysis demonstrated here. Another field of potential applications may be the analysis of alternative splicing, when cDNA should serve as the test template of amplification. The strategies tested demonstrate the versatility of flow cytometric microbead assays and further extend the realm of their possibilities. The assays described using a model system may find their application in HTP screening procedures directed toward specific genetic conditions, as well as provide a flow cytometric platform for molecular biological DNA analysis.

6. Summary

By the colony-forming test, I confirmed that the chromatin fragmentation phenomena observed reflect features of ~ 100 % viable cells,.

20-200 kb fragmentation of agarose embedded deproteinized S. cerevisiae and S. pombe chromatin derived from logarithmic or stationery cultures were observed after S1 nuclease digestions and urea/heat- or alkaline denaturation, establishing that there are ss discontinuities marking loop-sized fragments in the genome of both yeast strains.

I have not determined differences in the DNA fragmentation patterns of cells synchronized in G1-, S- or G2 phases.

I have established that the loop-sized fragmentation can be detected in all of chromosomes of S. cerevisiae or S. pombe after S1 nuclease digestions and urea/heat-denaturations.

Nicks and ARS sites exhibited an overall colocalization on chromosome I of S. cerevisiae.

The ~1.5 Mb rDNA cluster containing 100 -200 units of 9.1 kb showed loop-sized fragmentations similar to total DNA, i.e. nicks occur in every 11. unit on the average.

The nicks in the rDNA units were mapped by Southern blotting using rDNA specific probes. One of the nicks coincides with a region of RFB.

I have developed a new method called “Ab-Southern” to detect discontinuities and RNA/DNA hybrids in DNA molecules.

New gelelectrophoretic and flow-cytometric microbead assays have been established as part of my Ph.D. work.

7. Publications:

Related in extenso publications:

Hegedus, E., Kokai, E., Kotlyar, A., Dombradi, V., and Szabo, G., Separation of 1-23-kb complementary DNA strands by urea-agarose gel electrophoresis, *Nucleic Acids Res, 37*, e112 (2009). **IF: 6,878**

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Hegedus,E., Szekvolgyi,L., Kokai,E., Vereb,G., Bacso,Z., Dombradi, V., and Szabo, G., Loop-size chromatin domains resolved at the individual chromosome level. (*in preparation*)

Futher publications:

Szekvolgyi, L., Imre, L., Minh, D. X., Hegedus, E., Bacso, Z., and Szabo, G., Flow cytometric and laser scanning microscopic approaches in epigenetics research, *Methods Mol Biol, 567*, 99 (2009).

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Szilagyi, I., Varga, T., Szekvolgyi, L., Hegedus, E., Goda, K., Kaczur, V., Bacso, Z., Nakayama, Y., Posafi, J., Pongor, S., and Szabo, G., Jr., Non-random features of loop-size chromatin fragmentation, *J Cell Biochem, 89*, 1193 (2003). **IF: 2,664**

Cumulated impact factor: 18,136

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