Ph.D. THESIS

The role of persistent nicks at the boundaries of interphase chromatin loops and their possible involvement in pathological gene rearrangements

Lóránt Székvölgyi

Supervisor: Gábor Szabó M.D.Ph.D. D.Sc.

UNIVERSITY OF DEBRECEN
MEDICAL AND HEALTH SCIENCE CENTER
FACULTY OF MEDICINE
DEPARTMENT OF BIOPHYSICS AND CELL BIOLOGY
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1. INTRODUCTION

Regarding the spatial organization of the genetic material, it seems evident that the dimensions of the nucleus ($1.13x10^2$ μm^3 in humans) are vastly exceeded by the exceptionally long DNA molecule (1.8 m in humans), assuming a general packaging mechanism that follows a yet partially identified principle. The eukaryote genome is highly compartmentalized, starting from the "beads on a string array" of nucleosomes to the formation of metaphase chromosomes representing the highest level of compaction; packaging ratio (i.e. the ratio of the DNA double helix length to the actual length of the compartment that contains it) increases by many-fold during this process. At a certain level of hierarchical DNA packaging, as demonstrated by numerous studies, the 30 nm fibers fold up into repeating ~20-200 kbp loop domains, probably representing the fundamental units of both DNA replication and transcription. Research aimed at answering the question how chromatin fibers confined into a highly limited volume are involved in the coordination of biochemical processes and individual gene functions has gained impetus with the discovery of nuclear matrix/scaffold/nucleoskeleton. Based on advanced EM-visualization studies, the matrix appears to be built on a fine web of 10-nm matrix filaments that is associated with a second layer of nucleic acid containing structure, a fibrogranular RNA / ribonucleoprotein (RNP) network A large body of data suggests that this hnRNA-rich internal matrix might be also involved in anchoring the structural / functional units of higher-order chromatin structure, the supercoiled chromatin loop domains.

Indicative of the loop arrangement of higher-order chromatin organization, various chromatin fragmentation phenomena have been observed that involve the preferential cleavage of DNA, most likely at the bases of the loops, to ~20-200 kbp fragments. The phenomenon seems particularly important because *(a)* it reflects regularities in the organization of interphase

chromosomes, and *(b)* it appears to be interrelated with questions concerning e.g. apoptosis, genome instability and chemotherapy-associated gene rearrangements. Apoptosis is characterized by the degradation of DNA into a specific pattern of high and low molecular weight fragments; unequivocal explanation as to the mechanism of DNA degradation has yet to emerge. High molecular weight (HMW) DNA fragmentation involes the release of chromatin loops through the cleavage of DNA domains at S/MARs, whereas smaller fragments (the DNA ladder) arise from endonucleolytic activity at the linker regions. Until recenty, the executional phase of apoptosis has been perceived as an irreversible point with no turning back, committing the cells to die. Several recent observations seem to contradict these findings, offering a new model of carcinogenesis alluded to as 'abortive apoptosis'. This model proposes a mechanism whereby a special family of proteins, known as IAP's (Inhibitor of Apoptosis Proteins) specifically blocks the activated forms of caspase 3, a critical component of apoptosis execution. Cells arrest in the phase of HMW DNA fragmentation that may lead to incorrect DNA repair with rearrangements, and consequent cell recovery. This failure in the cell death program can leave cells with potentially tumorigenic DNA lesions causing serious clinical consequences. Candidate examples of a disease process related to the failed execution of apoptosis are the therapy related leukemia's such as Acute Lymphoid Leukemia (ALL) and Acute Myeloid Leukemia (AML).These diseases are linked to treatments of primary cancers utilizing inhibitors of topoisomerase II. The primary molecular alterations in leukemogenesis involving topo II activity have been intensively studied in a confined, 8.3 kbp-long breakpoint cluster region (bcr) within the *M*ixed *L*ineage *L*eukemia (*MLL*) gene. This bcr is also a predilection point for DNA cleavages early on in apoptosis, linking an error-prone repair of these breaks to pathological gene rearrangements. The connection between etoposide toxicity, DNA breaks and the regulatory machinery involved in

the control of chromatin structure at MLL bcr is generally recognized, but not clearly understood.

In the early '90s, our group discovered a new type of chromatin fragmentation that took place in isolated nuclei of healthy, proliferating eukaryotic cells upon treatment with ionic detergents[67]. The *en masse* disintegration of chromatin into regular, ~50 kbp fragments seemed unique in a sense that it was manifested in the absence of apoptosis and it involved almost the total nuclear DNA. Analysis of cloned breakpoint sequences revealed certain regularities suggestive of specific mechanisms that may account for the apparently non-random nature of fragmentation. It was also notable that one of the sequence motives shared by several ~50 kb breakpoints was also present in the topoisomerase II binding consensus within the breakpoint cluster region of the MLL gene.

The above findings remind of the apoptotic HMW fragmentation; however, they involve normal, non-apoptotic cells. Our concept, anticipating preformed ss DNA discontinuities in the chromosomes at loop-size intervals is distinguished from the prevailing view contemplating HMW chromatin fragmentation merely as a prelude to cell death. We hypothesize that ss breaks present at every ~50 kbp throughout the entire genome might explain the frequent involvement of loop anchorage sites in pathological gene rearrangements. Consequently, the elucidation of mechanism involved in these phenomena will be of great value to understand the link beween etoposide-evoked anomalies of DNA integrity in apoptosis and the molecular machinery organizing higher-order chromatin structure.

2. SPECIFIC AIMS

The four *major goals* of this study have been as follows**:**

1. We wished to detect possible single-stranded regions as predilection points of ~50 kbp fragmentation at the level of individual cells, implementing a novel experimental strategy based on a combination of cell-biophysical and molecular biological approaches.

2. We wished to characterize *in situ* the chemical nature of the revealed DNA-termini as well as their possible interaction with nuclear matrix components, with a special emphasis on the intranuclear topography of the breakpoints.

3. We wished to establish an *in vitro* and *in vivo* experimental system to better understand the primary molecular steps of leukemogenesis that may involve topo II activity. As a model, we have studied a prototypic fragile site, the breakpoint cluster region of MLL gene.

4. Having recognized the role of epigenetic regulation in the above phenomena, we attempted to develop a novel screening method with high-throughput potentialities for the clinical investigation of epigenetic markers.

3. MATERIALS AND METHIODS

Cells: Jurkat, ML-1, HL-60, Hela, NIH 3T3, DC3F and DC3FOH cells used in this study were cultured in standard conditions. Peripheral blood lymphocytes (PBLs) were freshly prepared using magnetic beads (Dynal) according to the manufacturer's recommendations.

Isolation of genomic DNA from mammalian cells: Isolation of genomic DNA was carried out as follows: cells were washed twice in PBS then lysed in lysis-buffer (50 mM Tris-Cl pH 8, 10 mM EDTA, 100 mM NaCl, 1 % SDS, 0.5 mg/ml proteinase K) at 55 °C for 12 hours. SDS/EDTA/Proteinase K lysates were directly applied for FIGE, or phenol/chloroform extracted DNA was proceeded by linear amplification analysis.

Neutral and denaturing field inversion gel-electrophoresis (FIGE): 1 % agarose gels were prepared in 1×TAE (40 mM Tris-acetate, 1 mM EDTA). Neutral field inversion gel-electrophoresis was carried out at 6 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. After gel-electrophoresis, gels were stained with 0.5 µg/ml ethidium bromide (EBr). Denaturing field inversion gelelectrophoresis was carried out as follows: agarose plugs containing intact human chromosomes were equilibrated in 1×TAE containing 8 M urea, for 2 hours, then cut into 2 equal parts. Heat denaturation was performed at 60°C, 80°C or 90°C for 5 or 15 minutes. The denatured DNA in these samples stay single-stranded (ss) during electrophoresis in 1×TAE containing 1 M urea, 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, when it cooled below 60°C. 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.

In vitro cleavage assays of the MLL bcr: High quality nuclear extracts were prepared with the nuclear extract kit of Activemotif, according to the manufacturer's instructions. Cleavage assays were carried out as follows: 2-3 µg pMEP4-MLL plasmid (from Peter Aplan, NIH, Bethesda, USA) was incubated in 20 µl buffer A (150 mM KCl, 15 mM Tris-Cl pH 7.4, 2 mM DTT, 10 mM $MgCl_2$) supplemented with 5 µl nuclear extract (and 40 µM etoposide (Sigma), where indicated), at 37 °C for 20 min. Proteins were removed by digestion with 200 µg/ml proteinase K (from MBI Fermentas) in the presence of 1 % Sarkosyl (Sigma), at 55 °C for 1h. Cleavage products

were phenol/chloroform extracted, ethanol precipitated and dissolved in 20 µl TE, then run on an 1% agarose gel in the presence of EBr or amplified by linear extension. In some experiments buffer A was omitted and the nuclear extract alone was added to the DNA samples. Size-fractionated nuclear extracts were generated by Superose gel chromatography (Amersham Pharmacia) following the manufacturer's recommendations; fractions were eluted in 500 µl buffer A, than tested for cleavage activity. For S1-nuclease sensitivity assays, 2 µg of pMEP4-MLL plasmid was cleaved in a volume of 100 µl by 2-25-100 U of S1-nuclease at 17 °C for 1 hour. Cleavage products were proteinase K treated, phenol/chloroform extracted, ethanol precipitated and resuspended in 20 µl TE, then run on a 0.5 % agarose gel or amplified by linear extension. Topoisomerase II-depleted nuclear extracts were made by *in vitro* immunoprecipitation with polyclonal antibodies against topo IIα (18511α) and topo IIβ (18513β) raised in rabbits. 10 µl of anti-human topoisomerase II polyclonal antibody was added to the mixture of 5 µl nuclear extract and 40 µl buffer A, and gently rotated for 3 hours on ice. Blocked protein A Sepharose beads (Upstate) were used to remove topoisomerase II-antibody complexes; after centrifugation, the supernatant was incubated with the pMEP4-MLL plasmid and amplified cleavage products were analyzed on a sequencing gel.

Linear amplification of the cleavage products: Linear amplification was based on the method published by Salus and Jost[76] with minor modifications. Briefly, the reaction was carried out in a volume of 100 µl in the presence of 0.5 µg pMEP4-MLL plasmid DNA treated as described above, or 25 µg BamH I digested genomic DNA. Linear PCR was carried out in samples containing 3 mM $MgCl_2$, 2.5 mM of each dNTP, 25 pmole sense or antisense primer (tgaatctcccgcaatgtcca and cttgaagggctcacaacagacttgg, respectively), 1 µl of deoxycytidine 5'-[α-^{32}P] triphosphate (3000 Ci/mmol, IZINTA Trading Co., Hungary), 3 U of Taq-polymerase (MBI Fermentas). After 30 cycles, the reaction products were phenol/chloroform extracted, ethanol precipitated and resuspended in 10 µl of 50 mM NaOH/0.5 M EDTA/4 M urea, 0.02 % xylene cyanol). Sequence ladders were generated with the fmol® DNA Cycle Sequencing System (Promega); 4 fmole of the PCR product was used as a template. The sequencing gels contained 8 % polyacrilamide/7 M urea. The samples were heated at 95 °C for 5 minutes immediately before loading and sequencing ladders were run alongside the amplified cleavage products. The results were evaluated on a phosphorimager (Biorad).

Nuclear halo preparation: Exponentially growing Jurkat and ML-1 cells, or freshly prepared peripheral blood lymphocytes (PBLs) were embedded in 1 % LMP-agarose and pipetted onto the surface of agarose-precoated

glass slides. Where indicated, Jurkat and ML-1 cells treated with 40 μM etoposide for 6 hours were used to investigate apoptotic DNA fragmentation. The samples were treated with a series of different lysis buffers for 6 minutes on ice (buffer A: 2 M NaCl, 10 mM Tris-Cl pH 8, 50 mM EDTA, 0.5 % Triton X-100, 10 % DMSO; buffer B: the same as buffer A, but with 0.5 M NaCl; buffer C: 2 M NaCl, 10 mM Tris-Cl pH 8, 50 mM EDTA, 0.2 % Sarkosyl, 0.5 % Triton X-100, 10 % DMSO; buffer D: the same as buffer C, but with 0.5 M NaCl). In certain experiments alkaline lysing conditions (pH 12), expected to preclude any enzymatic activity, were applied. Where indicated, the nuclear halos were fixed in 2 % formaldehyde for 10 minutes. All solutions contained protease inhibitors (Protease inhibitor cocktail, Sigma Aldrich). Loop-size DNA fragmentation was visualized by the comet assay, with the samples run in field inversion mode (FIGE-comet). FIGE of the nuclear halos and of isolated DNA was performed using an MJ Research PPI 200 power inverter; after running, the samples were neutralized in 1 M Tris-Cl pH 8 / 10 mM EDTA for 5 minutes, then dehydrated in an ascending series of ethanol for 2 minutes each at 4 °C, finally in methanol and acetone for 15 minutes each at -20 °C. Running parameters were set to maximize resolution in the ~50-400 kbp range. In the experiment shown in Figure 1.D., the nuclear halos and FIGE-comets were scraped off from the surface of the glass-slides and following β-agarase digestion, the DNA was isolated by phenol-chloroform extraction and analyzed by FIGE on a 1 % agarose gel, prepared in 1xTAE.

Preparation of chromatin fibers: Stretched DNA fibers were prepared exactly by the method described by Parra[77]. Briefly, Individual Jurkat cells or PBLs (100-3000 / slide) in 5 μl of PBS were placed at one and of a glass slide, air-dried and immediately lysed with a solution of 0.5 % SDS / 50 mM EDTA / 200 mM Tris (pH 7.4) for 5 minutes at room temperature. The slide was tilted to allow the drop of sample to run downwards by hydrodynamic forces, resulting in a DNA stream extending down the slide. The DNA stream was air dried and (1) in situ nick translated with DNA polymerase one, then fixed with methanol / acetone, or (2) first fixed in methanol /acetone, then in situ nick translated. The two methods gave comparable results.

Laser scanning cytometry (LSC): FIGE-comets were quantitatively evaluated by image analysis applying an iCys laser scanning cytometer and an iCyte 2.6 software (CompuCyte, USA). Cyber Gold stained samples were excited with the 488-nm argon-ion laser line of the equipment and the fluorescence emission was detected in the green channel applying a 530±15-nm band pass filter. Two different intensity level contour thresholds were set up for the green fluorescence. The first was set up to detect low level fluorescence intensity for whole comets.

The other contour threshold using a virtual channel was set up to recognize only the higher intensity nuclear locations for comet heads. Both the comet head and whole comet fluorescence intensity integrals and image locations were recorded. These primary data were transported into a Microsoft Excel sheet (Microsoft Corp. USA) and tail moment calculations were completed with the software. Tail moment is defined as a product of the distance between the head and tail mass centers and the relative amount of DNA in the tail compared with the total DNA in each comet, according to the following equation: TM = d x FLtail / (FLhead + FLtail), where FLhead is the DNA fluorescence in the comet head, FLtail is the DNA fluorescence in the comet tail, and d is the distance between the centers of mass of the head and the tail images. The unit of T is length[78,79].

Characterization of the DNA-termini by *in situ* nick translation: The presence of endogenous nicks in the nuclear halos was studied by situ nick labeling performed with DNA polymerase I, Klenow enzyme and terminal transferase, respectively. All reagents were purchased from Fermentas. Immediately after halo-preparation, the slides were washed in 100 ml of ice cold 0.5xTE for 5 minutes, and then 200 ul of labeling mix A (1x Pol I / Klenow buffer, 50 uM biotin-11-dUTP, 50 uM 3dNTP, 5 U DNA polymerase I / Klenow fragment) or mix B (1xTdT buffer, 150 uM biotin-11-dUTP, 5 U TdT) was added to each slide, respectively, and incubated under coverslips for 1-10 minutes at 20 °C. The reactions were stopped by washing in 100 ml of ice-cold TE for 10 minutes, and then dehydrated and fixed as described above. Biotin-11-dUTP incorporation was detected by monoclonal anti-biotin antibodies (Sigma), and the signals were enhanced by an AlexaFluor 488 labeled anti-biotin signal amplification kit (Molecular Probes).

Microscopy and digital image processing: Full field images were made with a 7.1 million pixels Olympus C-7070 WideZoom professional digital compact camera mounted on an Olympus CX31 microscope with 4X, 10X, and 40X achromat objectives, 50W Hg lamp light source from a reflected light fluorescence attachment and mirror units for blue and green fluorescence excitation. CLSM images were made by an LSM510 confocal laser scanning microscopy system. SybrGold stained samples were excited by the 488 Argon ion laser line, and emission was detected in single track mode, through the 515-540 band pass filter in the green fluorescence channel, applying 1.5 µm pinhole. Fluorescence signals of AlexaFluor 488-labeled samples were detected as described above, except that multi-track mode was used to simultaneously detect propidium iodide fluorescence through the 515-545 nm band pass filter of the red channel. Images were processed as follows: the size of chromatin fragments was estimated by dividing the length of diploid genome ($6x10^9$ bp) by the number of

fragments generated in alkaline FIGE or by the number of nick-translated spots. These numbers were determined by dividing the total (background corrected) fluorescence signal (PI stained DNA after FIGE or AlexaFluor 488 fluorescence of nick-translated spots) by the mean intensity of spots. Full field fluorescence images were processed using SCIL Image (TNO, Delft, The Netherlands). Binary masks to identify individual spots were created by thresholding the fluorescence images after background subtraction using the rolling ball algorithm. Lower threshold values were determined as average fluorescence from cell-free areas of the image; small objects originating from background noise were removed using another binary mask after Gauss filtering; spots were void of holes as confirmed by a dilation-erosion cycle. The size-distribution histogram of unit spots was generated and its median determined; the average fluorescence of median sized spots was taken as the mean intensity of individual spots which was of the same magnitude, within 20% error, as the median of spot fluorescence intensity distribution.

FISH analysis of nuclear halos and FIGE-comets: The DNA of nuclear halos and FIGE-comets were visualized by Sybr Gold (Molecular Probes), propidium iodide (Sigma) and DAPI (Vector Laboratoratories, Inc., USA), respectively. Fluorescence in situ hybridization was performed on nuclear halo and FIGE-comet slides prepared in mild lysis conditions (nuclear halo buffer A or buffer B), to completely preserve matrix and chromosome territory organization. To study loop-size DNA fragmentation at the MLL bcr, the LSI MLL Dual Color, break apart rearrangement probe (Vysis Inc., USA), spanning a contiguous 540 kbp genomic region of 11q23, including the breakpoint cluster region of the MLL gene, was used. The LSI MLL probe consists of a 350 kbp centromeric flank of the MLL bcr labeled with SpectrumGreen, and a 190 kbp telomeric flank of the 8.3 kbp MLL bcr labeled with SpectrumOrange, sonicated to an average fragment size of 600 bp. The chromosome 8 specific probe was generously supplied by the Resource for Molecular Cytogenetics (University of California Cancer Center, San Francisco, CA, USA) and labeled by biotin via nick translation according to the protocol of the supplier (ROCHE Diagnostics GmbH, Applied Sciences, Germany). The chromosome 15 painting probe was purchased from Appligene and was used according to the manufacturer's recommendations. The slides were denatured in 2 M NaOH (2 min, at room temperature), dehydrated in an ascending series of ethanol (70%, 85%, 100%; 2 min each), and air dried. DNA probes and cellular DNA were denatured at 73°C for 5 min, and the probe solution was dropped on the cells. Overnight hybridizations were performed at 37 °C in a hybridization oven. Post-hybridization washing steps were done according to the manufacturer's instructions. Samples were scored for the number of fluorescent signals per cell using a fluorescent microscope (OPTON, Oberkochen,

Germany) equipped with selective filters for the detection of FITC, SpectrumGreen, SpectrumOrange and DAPI. Three colour images were captured using a digital imaging analysis system (ISIS Metasystem GmbH, Altlussheim, Germany).

Chromatin immunoprecipitation: All reagents used contained protease inhibitors (Protease inhibitor cocktail, Sigma Aldrich). ChIP was performed according to the method published by Kuo and Allis[80] with modifications: Jurkat cells were cultured in standard conditions, and where indicated, they were treated for 3 hours with 40 µM etoposide (Sigma) to induce apoptosis. Cells were fixed with 1 % formaldehyde for 10 minutes at room temperature, and fixation was stopped by the addition of glycine to a final concentration of 150 mM. For the isolation of nuclei, cells were washed twice in ice-cold PBS, and incubated for 10 minutes in buffer N (5 mM Pipes pH 8, 85 mM KCl, 0.5 % NP-40) on ice. After centrifugation at 3000 g at 4 °C for 10 minutes, the pelleted nuclei were resuspended in sonication buffer (1 % SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8), incubated on ice for 10 minutes, then sonicated to an average fragment size of 200 bp. Cell debris were removed by centrifugation at 10000 g at 4 °C, for 20 minutes; aliquots of soluble chromatin were frozen in liquid nitrogen and stored at -80 °C. Before immunoprecipitation, 0.1 ml of the chromatin solution was diluted 10-fold in buffer IP (0.01 % SDS, 1.1 % Triton X-100, 1.2 mM EDTA, 20 mM Tris pH 8, 167 mM NaCl), and the samples were precleared with 40 µl of blocked protein A Sepharose beads (Upstate, catalog no. 16-157). Immunoprecipitation was carried out overnight on a rotating plate at 4 °C with antibodies (Upstate) against modified histones: anti-acetyl H4, 2 µg/IP, catalog no. 06-866, and anti-dimethyl H3 Lys 4, 5 µg/IP, catalog no. 07-030. Complexes were collected using 40 µl of blocked protein A Sepharose, and an aliquot of the 'no antibody' control sample was preserved to determine the amount of amplified DNA obtained without immunoprecipiation ('input' DNA). Subsequently, the Sepharose beads were pelleted and washed twice with each of the following buffers: buffer A (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl; pH 8), buffer B (0.1 % SDS, 1 % Triton-X 100, 2 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl; pH 8), buffer C (0.25 M LiCl, 1 % NP 40, 1 % sodium deoxycholate,1 mM EDTA, 10 mM Tris-HCl; pH 8), and TE (10 mM Tris-HC, l mM EDTA; pH 8, 1). Cross-links were reversed by heat-treatment at 65 °C for 6 hours, then samples were treated with 0.2 mg/ml Dnase-free RNase (from Sigma Aldrich) for 30 minutes at 37 °C. Proteins were digested by 0.5 mg/ml proteinase K in the presence of 0.1 % SDS for 3 hours at 50 °C and DNA was purified on PCR clean-up columns (Qiagen). Samples were eluted in 50 µl of TE and stored at -20 °C. The level of histone acetylation and methylation were determined by *a)* the ChIP-on-beads technique, *b)* real time quantitative PCR. Both in the case of 'no antibody' (nAb) controls and 'Ab' samples, the number of copies were expressed as a percentage of input DNA.

PCR and real time quantitative PCR (QPCR): PCR reactions were carried out in reaction volume of 25 µl. Primers designed for *(a)* the breakpoint cluster region of the MLL gene: fw5'-3' AGTCTGTTGTGAGCCCTTCCA, rev5'-3' CGACGACAACACCAATTTTCC, probe5'-3' Fam-AAGTTTTGTTTAGAGGAGAACGAGCGCCCT-Tamra (termed 'telomeric assay'), fw5'-3'-TGCAGGCACTTTGAACATCCT, probe5'-3 Fam-AGCACTCTCT CCAATGGCAATAGTTCTAAGCAA-Tamra,' rev5'-3' CTGATCCTGTGGACTCCA TCTG (termed 'centromeric assay'), and *(b)* for the promoter region of the human TGM2 gene investigated in ChIP-on-beads studies: fw 5'*Fam*-GAGACCCTCCAAGTGCGAC-3', rev 5'*Biotin*-CCAAAGCGGGCTATAAGTTAGC-3'. Standard reaction conditions were applied (2,5 mM dNTP, 0,4 µM of forward and reverse primers, 1.5 mM $MgCl_2$, 2 U Taq polymerase, 2 µl of ChIP-DNA) with a two-step cycling profile: 1x 95 °C:1 minute; 24x 95 °C: 12 sec, 55 °C: 30 sec; 1x 60 °C: 7 minutes. In the ChIP-on-beads studies, *Fam*/*biotin* labeled products were purified on PCR clean-up columns, and then analyzed by either agarose gel-electrophoresis, or by flow cytometry, capturing them on streptavidin-conjugated microbeads (see below). The PCR reactions were stopped in the linear phase as validated by real time QPCR measurements, that were performed by the same but unlabeled oligonucleotides, in conjunction with a TaqMan probe of 5'-3' Fam-CCGCCTCGGCAGTG CCA-Tamra. All reactions were performed in a volume of 22 µl according to the manufacturer's instructions. Measurements were done in triplicates, and the relative number of amplicons was determined by the $2^{-\Delta Ct}$ quantification method. The MLL and TGM2 mRNA levels were measured by reverse transcriptase real time QPCR as described. Total RNA was extracted by Trizol reagent (Invitrogen) from control and etoposide-treated Jurkat and ML-1 cells as decribed[81]. The mRNA copy numbers were normalized to cyclophilin transcript levels.

Measurement of DNase I sensitivity: Nuclei were prepared from Jurkat and ML-1 cells resuspended in SSCP (0.15 M NaCl, 0.015 M cacodylic acid), by lysing them in ice-cold SSCP + 0.25 % NP-40. Five volumes of ice cold Hepes-buffer (0.17 M KCl, 10 mM Hepes, 3 mM $MgCl_2$) was added, and the samples were centrifuged. Nuclei were recovered in Hepes-buffer and divided into several aliquots, which were treated with different concentrations of DNase I (Promega) for 10 minutes at room temperature. Digestion was stopped by incubation of the nuclei resuspended in lysis-buffer (50 mM Tris-Cl pH 8, 10 mM EDTA, 100 mM NaCl, 1 % SDS, 0.5 mg/ml proteinase K) at 55 °C for 16 hours. DNA was isolated by phenol-chloroform extraction and ethanol precipitation, dissolved in TE buffer, digested with BamH1 and re-extracted as above. The number of intact

MLL-copies in each sample was determined by real time QPCR and normalized to the control without DNase digestion.

Flow cytometry:

Cell cycle measurements: normal and etoposide-treated Jurkat and ML-1 cells were fixed in ice-cold 70 % ethanol at -20 °C, overnight. After fixation, cells were resuspended in PBS, and treated with 100 µg/ml RNase A for 30 minutes at room temperature. DNA was stained with propidium-iodide at a final concentration of 50 µg/ml. Measurements were carried out on a Becton-Dickinson FACScan flow cytometer (Mountain View, CA, USA): the samples were run at *high speed*, the laser power was set to 15 mW and the fluorescence signals were detected through the FL2 channel, in logarithmic mode. The results were evaluated by the BDIS CELLQUEST 3.3 (Becton-Dickinson) software and the indicated conditions were applied for FISH and ChIP analyses (see Suppl. Fig. 5.B.). In some FISH experiments, S-phase cells were selectively labelled by a 1 hour pulse of BrdU. Washing steps and FISH analysis were carried out as described[82]. The BrdU-foci were detected by mouse-anti-BrdU and goat-anti-mouse-FITC antibodies (Vector Laboratoratories, Inc., USA), used at a dilution of 1:50 and 1:500, respectively. Incubations and subsequent washes were done as described.

Measurements of epigenetic changes by ChIP-on-beads method: immunoprecipitated DNA samples (input, nAb, Ac H4/Met H3 K4, respectively) were tagged with 5'-*Fam/biotin* by PCR. 5 µl of tagged ChIP-DNA was added to 10,000 streptavidin-coated, plain beads (purchased from Polyscience AG, Switzerland) in 50 µl PBS. These samples were incubated for 20 minutes at room temperature, and then washed in 1 ml PBS. Flow cytometry was performed as described above, except that fluorescent signals were collected thorugh the FL-1 channel.

4. RESULTS AND DISCUSSION

Although the higher-order structure of eukaryotic chromosomes has been the focus of considerable attention, little is known about the signals that mark the boundaries of interphase chromatin loops. Applying biophysical and molecular biological techniques, we have visualized the global disassembly of the chromatin of healthy, non-apoptotic human cells into particles apparently containing the DNA-loops, for the first time. Data we have obtained provide evidence for the existence of preformed single-strand discontinuities all over the entire genome, which appear to be arranged on the two DNA strands in an alternating, staggered fashion, positioned at loop-size intervals. The chemical nature of the DNA-termini have been characterized by in situ nick labeling, revealing uniform ends with 3'OH groups, tightly protected by structures sensitive to ribonucleolytic treatments. Our results suggest an association between the nicks present at the bases of chromatin loops and the nuclear matrix attached architectural hnRNA-network.

To study the role of particular DNA regions in the observed phenomena, we extended our studies on the breakpoint cluster region (bcr) of the *M*ixed *L*ineage *L*eukemia (MLL) gene that is frequently rearranged in childhood and posttherapeutic leukemias. The sequence specificity of nick accumulation, the possible function of nick froming sequence motives as well as the role of topoisomerase II have been investigated by linear primer extension footprinting and chromatin immunoprecipitation (ChIP). The results demonstrate the involvement of the above enzyme in the generation / maintenance of nicks at non-random positions. As studied by halo-FISH and ChIP, the disintegration of the chromatin at MLL bcr was dependent on local epigenetic and broader chromosomal context, with marked differences between germline and rearranged MLL. The high level of H3K-acetylation and H3K4-methylation at gMLL indicates transcriptionally active, relaxed chromatin structure, suggesting that the incidence of ss-breaks is highly dependent on chromosomal context. We

hypothesize that the ss discontinuities present at every ~50 kbp throughout the genome might explain the frequent involvement of loop anchorage sites in chromosomal translocations.

In view of the special significance of epigenetic context in the incidence of nicks - probably predisposing for pathological gene rearrangements, we have also developed a novel high-throughput screening method for the evaluation of ChIP results, applicable in a clinical set-up, combining biophysical and molecular biological know-how.

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5. PUBLICATIONS

THE THESIS IS BASED ON THE FOLLOWING IN EXTENSO PUBLICATIONS:

(1) Szekvolgyi L, Hegedus E, Molnar M, Szarka K, Beck Z, Dombradi V, Austin CA and Szabo G. Nick-forming sequences may be involved in the organization of eukaryotic chromatin into approximately 50 kbp loops. *Histochem. Cell Biol.* (2006) **125:** (1-2): 63-73, **IF: 2.594**

(2) Szekvolgyi L, Balint LB, Imre L, Goda K, Szabo M, Nagy L and Szabo G. ChIP-on-beads: flow-cytometric evaluation of chromatin immunoprecipitation. Accepted for publication in *Cytometry Part A*, **IF: 2.698**

SUBMITTED MANUSCRIPT THE THESIS IS BASED ON:

(3) **Szekvolgyi L**, Rakosy Zs, Balint LB, Bacso Zs, Goda K, Vereb Gy, Varga S, Balazs M, Nagy L and Szabo G. Preformed nicks mark the boundaries of interphase chromatin loops.

FURTHER IN EXTENSO PUBLICATIONS:

(4) Pataki J, Szabo M, Lantos E, **Szekvolgyi L**, Molnar M, Hegedus E, Bacso Zs, Kappelmayer J, Lustyik Gy and Szabo G. Biologycal microbeads for flow-cytometric immunoassays, enzyme titrations and quantitative PCR. *Cytometry Part A* (2005) **68A**: 45-52, **IF: 2.698**

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