SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Accessibility of DNA in chromatin: The role of constrained superhelicity

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DEBRECEN, 2021

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The Examination took place at the Discussion room of the Immunology Department, Faculty of Medicine, University of Debrecen, at 2 pm on 6th June 2018.

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The PhD defense will take place on 29th October, 2021 at 10:00 am via the Zoom online meeting platform. Public participation is guaranteed.

1. INTRODUCTION

1.1 ACCESSIBILITY AND TRANSCRIPTIONAL REGULATION

Transcription regulation is necessary for cell differentiation and for maintaining cell-type specific phenotypes, and its disorders often lead to disease. In the cell, transcription occurs in the context of chromatin which is maintained in a repressive state by the nucleosome structure. Thus, transcription initiation requires the generation of nucleosome free regions (NFRs) to allow TF binding. Nucleosomes ahead of the transcribing polymerase also have to be remodelled/ destabilized to allow passage of the transcription machinery.

NFRs exhibit a 100-fold higher sensitivity to nucleases than the rest of chromatin and are thus termed nuclease hypersensitivity sites (NHSs). Further, majority of NHSs determined so far correspond to regulatory elements such as enhancers, promoters, insulators, silencers and locus control regions. Consequently, by combining nuclease hypersensitivity assays with next-generation sequencing (NGS) it is now possible to profile all the regulatory sequences genome-wide.

1.1.1 Nuclease-based DNA accessibility assays

DNase-seq, MNase-seq and ATAC-seq utilize enzymes that bind and cleave DNA in a non-sequence specific manner. A variant to the DNase-seq is FAIRE-seq which utilizes sonication to cleave DNA also in a non-sequence specific manner. The assays rely on two principles; 1) that DNA sequences bound by protein are protected from digestion or resist sonication and 2) that the histones are the main molecular species in contact with DNA. They thus provide a snapshot of the nucleosome occupancy and the accessible DNA genome-wide. This snapshot is a population average, since they use millions of cells and are only being adapted to single cell protocols recently. Nevertheless, there is a general overlap in the sequences identified as open across the assays.

1.1.2 Microscopy-based techniques for studying DNA accessibility

Accessibility of DNA in various chromatin domains has also been studied by microscopy-based techniques which track the mobility and distribution of inert fluorescent molecules microinjected

into the nuclei. These techniques have revealed that molecules up to ~80 kDa equally distribute in the nuclei, nucleoli exempted. Though fluorescence microscopy resolution is limited to 250 nm, these observations show that gene-specific TFs which are about 50 kDa can easily access their promoters even in heterochromatin. Further, dynamic chromatin motions render sites even in heterochromatin accessible through increase of the actual pore sizes or dynamic exposure.

Enzymatic assays for DNA accessibility present the nucleosome as a physical barrier to TF binding. This is corroborated by biophysical studies which rule out dense packaging as a barrier. Considering that the DNA is wrapped around the nucleosome with at least 50% of its surface facing the outside, reduced accessibility to nucleosomal DNA cannot be fully explained by steric hindrance.

1.1.3 Transcription factor binding specificity

General and gene-specific TFs recognize and bind to specific DNA sequence motifs in the cis-regulatory elements through the establishment of hydrogen bonds between amino acids on the DNA binding domain of the TF and the DNA bases. The sequence motifs recognized by TFs are about 6-10 bp and exhibit great sequence degeneracy. Sequence specificity for any particular TF can be determined by *in vitro* DNA binding assays such as protein binding microarrays & SELEX-based techniques, and these can be used to in turn predict transcription factor binding sites *in vivo*.

It is now also recognized that DNA structural features play a role in TF binding specificity and their inclusion to computer models improves binding site prediction. The features include the DNA minor groove width, DNA curvature and flexibility and parameters describing the inter-and intra-base pair spatial relationship. For example, DNA binding proteins with positively charged residues such as arginine and histidine on their surface are likely to be attracted to a narrowed minor groove due to its increased negative electrostatic potential.

The structural parameters of DNA can be modulated by a number of factors including i) the binding of an intercalator which locally unwinds DNA increasing the helix twist and also the rise between successive base pairs where they bind; ii) the binding of some structural proteins to DNA such as HMGB1 and histone proteins which cause severe bends and kinks to the local DNA structure; iii) DNA base mismatch which may widen (pyrimidine-pyrimidine) or constrict (purine-purine) the base pairs ; and iv) DNA supercoiling.

1.2 DNA INTERCALATORS

Intercalating molecules possess a characteristic electron-deficient planar ring system termed chromophore, which inserts between adjacent DNA base pairs. While simple intercalators such as ethidium, propidium and proflavin consist of only the chromophore, other intercalators have in addition peptide groups or sugar moieties. These additional groups non-covalently interact with bases in the DNA grooves and contribute to sequence specificity and the orientation of the bound ligand. Intercalation of the hydrophobic chromophores into DNA is energetically favoured since it removes the nonpolar ligand from the polar environment of water.

Intercalator binding to naked DNA has been extensively studied revealing that intercalation requires local base pair separation to accommodate the intercalator. The distance between adjacent bases increases by 3.4 Å per mono-intercalator and 6.8 Å per bis-intercalator molecule, thus increasing the length of the DNA duplex. Further, intercalation causes unwinding of the DNA in the base pairs adjacent to the site of intercalation. The degree of unwinding is dependent on the nature of the intercalator. For example, EBr unwinds the DNA by 26°, YOYO-1 by $\sim 24\pm8^{o}$ and anthracyclines by 11°. The local perturbation from intercalation however spreads along the DNA molecule.

Four intercalators were used in my research; ethidium bromide (EBr) and YOYO-1 (bisintercalator) which are cell membrane impermeant and are widely used as DNA dyes. The exhibit no sequence specificity and following intercalation, there fluorescence quantum yield is increased 20-fold for EBr and >1000-fold for YOYO-1. The other intercalators, doxorubicin (Dox) and psoralen are cell membrane permeant molecules of medical importance. Dox is a well-known antitumor antibiotic which preferentially binds between GC base pairs. Upon intercalation the Dox fluorescence is greatly quenched. Psoralen is a naturally occurring furanocoumarin used in the treatment of some skin disorders. When intercalated molecules are exposed to UVA, they form covalent adducts with thymine. Though psoralen absorbs UV and emits a weak blue light, for laboratory use it is commonly tagged with a functional group such as biotin for easy identification.

1.3 DNA SUPERCOILING AND NUCLEOSOMAL DNA

DNA supercoiling is a vector quantity made up of two mathematical properties, twist (*Tw*) and writhe (*Wr*). *Tw* describes the number of times one DNA strand winds about the other in a duplex, while *Wr* is the number of times the duplex winds about its own axis. Writhes that form in space are referred to as plectonemes, whereas those occurring around an object are referred to as toroids.

DNA is wrapped around the nucleosome 1.67 times in a left-handed fashion and would thus be expected to generate a linking number difference of ~-1.67. However, the experimentally determined ΔLk per nucleosome (ΔLk = -1.0) differs from theoretically expected one (ΔLk = -1.67). This difference is known as the *Lk* paradox and has been the subject of much research. Two main hypotheses supported by experimental evidence, have been put forward to explain the *Lk* paradox. One hypothesis postulates that the DNA wrapped around the core nucleosome is over-twisted by about + 0.23. The second hypothesis states that the length of the linker DNA determines its path, which in turn contributes to the ΔLk. Considering the experimental evidence supporting the two hypotheses, it is probable that the linking number paradox is only fully explained by taking into account both the twist changes in nucleosomal DNA and the contribution of the linker DNA length.

1.3.1 Superhelical constraint & deformation of nucleosomal DNA

The DNA winds around the histone octamer with its minor and major grooves alternately facing the octamer. The minor groove faces the nucleosome core at 14 different locations: SHL ±0.5, ±1.5, ± 2.5, ±3.5, ±4.5, ± 5.5 and ±6.5. It is at these sites that histone-DNA contacts are established in a sequence-independent manner. A total of 142 direct and at least 358 water mediated hydrogen bonds link the DNA to the histone octamer per nucleosome core particle (NCP). In addition, electrostatic interactions between the negatively charged phosphate DNA backbone and positively charged amino acids (Lys^+/Arg^+) in histones, as well as van der Waals interactions strengthen the grip on nucleosomal DNA. Though nucleosomal DNA undergoes spontaneous transient unwrapping from the ends, in steady state the DNA is largely constrained on the octamer by the large number of bonds. This constraint would be expected to greatly hamper the degrees of freedom for nucleosomal DNA.

In addition to the superhelical changes and the constrained conformation, the DNA in the NCP is greatly bent, such that 80 bp form a complete superhelical turn. This is a great deviation from the persistent length of linear B-DNA in solution which is 150 bp. This bending results in widening of both the minor and major grooves that face away from the octamer, while the minor grooves that face the octamer become narrow. Furthermore, an arginine residue penetrates into each of the inward facing minor grooves where it kinks the DNA.

1.4 THE HMG-BOX DNA BINDING DOMAIN & HMGB1 PROTEIN

The HMG-box is a 75 amino acid DNA-binding domain initially discovered in the architectural proteins HMGB in mammals, HMG-D in Drosophila and yNHP6A in S. cerevisiae. Later it was also found in chromatin remodelers such as BAF35 & BAF57, the nucleosome chaperone FACT, and transcription factors such as TFAM in the mitochondria, UBF1 in the nucleoli, the Sox family of developmental TFs, and the TCF/LEF-1 that regulate gene expression during differentiation, among others. This domain has been shown to preferentially bind supercoiled, cisplatin damaged DNA, hemicatenanes and other non-B DNA structures. It binds to the DNA minor groove causing unwinding and widening of the minor groove as well as bending of DNA towards the major groove. The bending of DNA is brought about by partial intercalation of amino acid side chains.

The architectural protein HMGB1 consists of 2 box domain and an acidic c-terminal tail. In the nucleus it binds to linker DNA in competition with histone H1. Following necrosis or activation of macrophages and monocytes, HMGB1 is release to the extracellular milieu where it acts as an alarmin. In living cells, it is actively shuttled between the nucleus and cytoplasm. Its activity and location is modulated by its PTMs.

HMGB1 has been described as a jack of all trades due to its involvement in many cellular processes including transcription, DNA repair, DNA recombination, chromatin organization and inflammation. It exerts its nuclear functions by i) directly restructuring the nucleosome or recruitment of nucleosome remodellers to make the DNA more; ii) DNA bending to enhance binding of TFs that require pre-bent DNA such as p53 and/or iii) directly interacting with TFs to increase their residence time on the promoters. It has also been shown to be recruited to DNA damage sites where it either enhances or obstructs repair.

2. JUSTIFICATION & OBJECTIVES

2.1 JUSTIFICATION

The nucleosome structure has long been known to be a barrier to regulatory factor binding *in vivo*, and this was generally assumed to be due to steric hindrance. This view is, however, challenged by the fact that DNA is wrapped on the outside of the nucleosome structure with one of its strands exposed at any one point. Further, biophysical studies have revealed that relatively large molecules can access even densely packaged chromatin areas and local chromatin Brownian motions can bring buried DNA targets for factor binging. Thus, there is need for further experiments to elucidate the mechanism by which the nucleosome structure inhibits binding.

The results obtained from my experiments with small molecule intercalators suggest that constrained superhelicity hampers intercalation into DNA in chromatin. Considering that some protein DNA interactions involve intercalating moieties, I wondered if superhelicity would equally affect their binding. HMGB1 is an example for such a protein, containing an HMG-box domain what has been identified in various proteins involved in transcription, chromatin remodelling and even DNA repair. The binding of this domain to naked DNA in solution has been extensively studied but information is rather scanty regarding its binding features in the cell's inner environment, *in vivo*, warranting further investigation.

2.2 OBJECTIVES

2.2.1 General objective

To determine the mechanism by which chromatin packaging regulates access to DNA *in vivo*.

2.2.2 Specific objectives

1. To develop an assay to evaluate the binding of small molecule intercalators *in situ*.
2. To determine the factors regulating intercalation of small molecules *in situ*.
3. To assess the influence of factors affecting DNA supercoiling on the binding of HMGB1, a protein with intercalating moieties, to DNA *in vivo*.

3. MATERIALS & METHODS

3.1 MATERIALS

All materials were obtained from Sigma Aldrich Hungary except where otherwise stated.

Cells and lines used: HPBMCs, HeLa, HeLa$^{H1c\text{-}GFP}$, HeLa$^{H2B\text{-}GFP}$, HeLa$^{H3\text{-}GFP}$ and U2OS^{2FP}.

3.2 METHODS

3.2.1 Cells and cell culture

HeLa cells were maintained in RPMI-1640 while HeLa$^{H1c\text{-}GFP}$, HeLa$^{H2B\text{-}GFP}$ HeLa$^{H3\text{-}GFP}$ and U2OS^{2FP} were maintained in DMEM medium (Gibco). Both RPMI and DMEM were supplemented with 10% FBS, 1x Glutamax, 100 U/ml penicillin, 100 µl/ml streptomycin and phenol red. In addition, media for U2OS^{2FP} cells were suplemented with G418 0.5 mg/mL for continous selection of transformed cells. All cells were cultured at 37°C in a humidified, 5% CO_2 incubator and passaged every two days. Cells were harvested using 1x Trypsin-EDTA following the standard protocol.

HPBMCs were separated from whole human blood obtained from healthy donors following the density gradient centrifugation technique developed by Böyum.

3.2.2 Live cell microscopy

Cells cultured overnight in an 8-well chamber (Ibidi, Martinstried, Germany) were rinsed in PBS and 300 µl of 10 µM ethidium bromide (EBr) in PBS-glucose added to the wells. Samples were imaged on the Olympus FV-1000 microscope. The microinjection experiment was carried out on a Zeiss LSM 5 Live (Carl Zeiss, Oberkochen, Germany) coupled with a patch clamp apparatus. Individual cells were microinjected in a whole-cell patch clamp set-up with a dye solution equivalent to that in the bathing solution. Patched cells were imaged for 20 minutes, every 30 seconds for the first 5 minutes and then every 2 minutes for the remaining time.

3.2.3 Embedding cells into agarose & nuclei preparation

The procedure of coating 8-well chambers, embedding live cells into agarose and nuclei preparation was carried as described by Imre and colleagues.

3.2.4 RNA digestion and salt induced histone elution.

All forms of RNA were digested using 150 µl/well of 100 µg/ml RNase A (Thermo Scientific, Waltham, Massachusetts, USA) dissolved in PBS-EDTA for 2 hr at 37°C. After RNA digestion, nuclei were washed with 400 µl/well of NaCl solutions, 6x for 10 min each. NaCl was washed from the wells using 400 µl /well of PBS-EDTA 3 x for 10 minutes each before addition of intercalators.

3.2.5 Intercalator application

Either 300 µl/well of 10 µM EBr or 150 µl/well of 40 nM YOYO-1 or 150 µl/well of 1 mg/ml biotinylated 4,5',8-trimethyl psoralen (bTMP) (kindly provided by Nick Gilbert) was added to nuclei and incubated for 2 hrs on ice. Unbound bTMP was removed by briefly rinsing the nuclei with PBS-EDTA. To induce bTMP-DNA cross links, the samples were exposed to UVA (365 nm) on ice for 10 min at a distance of 5 cm from the lamp. bTMP was detected by IF.

3.2.6 DNase I hypersensitivity assay

RNA-depleted, histone eluted nuclei were equilibrated with 400 µl/well of DNase I buffer (10 mM Tris pH 8, 0.1 mM $CaCl_2$ and 2.5 mM $MgCl_2$) then the DNA was nicked by DNase I digestion for 10 min at 37°C (300 µl/well of 0.1 µg/ml DNase I). Residual enzyme was removed or its activity inhibited by three washes with PBS-EDTA. The samples were then equilibrated with DNA polymerase I buffer (50 mM Tris-HCl, pH 7.2, and 10 mM MgSO4). Nicks generated by DNase I were labelled by *in situ* nick translation in a reaction volume of 120 µl/well consisting of 0.5 U of DNA polymerase I, 5 nmol of dATP, dCTP, dGTP and biotin-dUTP for 10 min at RT.

3.2.7 MNase Digestion

Formaldehyde fixed nuclei were equilibrated with MNase buffer (50 mM Tris, pH 7.5, 1 mM $CaCl_2$) and then digested with 150 µl/well of varying amounts of MNase at 37°C for 7 minutes. MNase activity was stopped by 3 washes with PBS-EDTA.

3.2.8 Immunofluorescence labelling

This applies to detection of psoralen, remaining histones, biotin dUTP and lamin B1. The samples were blocked with 1% w/v BSA in EDTA for 45 minutes then incubated with 150 µl/well of 1 µg/ml primary antibody overnight at 4 °C. Unbound primary antibody was removed by 3 washes with PBS-EDTA before a 2 hr incubation on ice with 200 µl/well of 1 µg/ml fluorophore conjugated secondary antibody.

In each case samples were counterstained with 300 µl/ well of 1 µg/ml (3.6 µM) DAPI for 2 hrs on ice before imaging with the laser scanning cytometer (LSC).

3.2.9 In-gel cell irradiation of cells and determination of nick incidence

Live cells were embedded into agarose plugs, DNA extracted, S1 digested and electrophoresed following the method described by Varga et al. However, immediately after embedding and prior to DNA extraction, plugs were submerged in culture medium and cells irradiated with varying doses of X-ray radiation at a distance of 57.2 cm from the 6 MeV linear accelerator. For nuclei experiments, live cells embedded in agarose in the wells of an 8-well chamber were irradiated with 300 Gy X-ray radiation as stated above.

3.2.10 Fluorescence lifetime imaging and data analysis

The fluorescence decay dynamics were acquired using a Nikon A1 laser scanning microscope with a PicoQuant time-correlated single photon counting module. Data was analysed using SymPhoTime 64 software and fitted using n-Exponential reconvolution module for 2 lifetime components.

3.2.11 Cell treatment for HMGB1 & histone H1 experiments

HeLa$^{H1c\text{-}GFP}$ and U2OS^{2FP} cells cultured on an 8-well chamber overnight were treated with the indicated concentrations of either doxorubicin for 2 hrs, EBr for 1 hr, bleomycin for 2 hrs or H_2O_2 for 20 min at 37°C in phenol red-free medium. Irradiation with X-rays was done as described above.

3.2.12 Strip FRAP

Strip FRAP measurements for histone H1-GFP were carried out on the Olympus FV-1000 microscope as described previously with minor modification. In brief, using a 60x, 1.2 NA oil immersion objective and a wide pinhole, 10 pre-bleach images (512x512-pixel, pixel size: 0.045 µm) of a cell were collected (488 nm laser line 4.5 µW pixel dwell time of 0.664 µs). A strip ROI (256 x12 pixels) was bleached (15x, 488 nm laser 55.6 µW pixel dwell time of 20 µs). 80 post-bleach images for a total time of 139.5 s. To standardize the geometry of the measurement, the FRAP ROI was positioned horizontally at one third of the vertical extension of the nucleus, avoiding nucleoli. Image fluorescence was analysed in Fiji ImageJ. Recovery of fluorescence in the ROI was normalized to the total fluorescence of the nuclei at each time point to take care of photobleaching. Recovery data analysis was using a one phase decay.

3.2.13 Point FRAP

Point FRAP measurements were performed on the Olympus FV-1000 microscope. EGFP was excited at 488 nm, and fluorescence was recorded in the 500–520 nm range. A confocal image of a cell (512×512-pixel, pixel size: 0.103 µm) was taken followed by the selection of a laser spot at which the laser beam was focused. Before bleaching, 5120 pre-bleach pixels were collected with a pixel dwell time of 10 µs (51.2 ms) followed by bleach period for 51.2 ms with 100% laser power 55.6 µW and then collecting 40,000 post-bleach pixels from the same spot for a total time of 297.59 ms.

In order to change the laser power of the Ar-ion laser in ~1 µs, a NI 7833 field programmable gate array (FPGA) card (National Instruments, Austin, TX) was used to control power switching together with a dedicated LabVIEW program which was developed (by Gábor Mocsár). Fluorescence recovery data were fitted assuming a one-component exponential recovery using a custom written Matlab (The Math Works, Natick, MA) program (by Gábor Mocsár).

3.2.14 Fluorescence correlation spectroscopy (FCS)

For FCS measurements, 1 µg of native pEGFP-C3 plasmid DNA and 945 ng of Alexa 647 labelled rHMGB1 were used per well in a volume of 300 µl of protein binding buffer with or without doxorubicin. The mixture was allowed to equilibrate at RT for 1 hr before measurement. FCS

measurements were carried out on a Nikon A1 Eclipse Ti2 confocal laser-scanning microscope (Nikon, Tokyo, Japan). Fluorescence autocorrelation curves were calculated by SymPhoTime64 software (PicoQuant, Berlin, Germany) at 200 time points from 300 ns to 1 s with quasi-logarithmic time scale. Autocorrelation curves were fitted to a model with triplet state and two diffusion components to account for DNA bound HMGB1 (slow component) and free 3D-diffusion of HMGB1 (fast component). 100 nM Alexa 647 dye (dissolved in 10 mM Tris-EDTA buffer, pH 7.4) with a known diffusion coefficient (D_{A647} = 330 $\mu m^2/s$, at T = 22.5°C) was used to determine the observation volume. All correlation curves were fitted using the free online QuickFit 3.0 software.

3.2.15 Expression and purification of rHMGB1

Expression of rHMGB1 (C23S, C45S, C106S and E204C) from pET19b expression vector was done in Rossetta (DE3)pLysS (plasmid &protocol kindly provided by Jennifer Kugel at University of Colorado Boulder, USA).

Samples were fluorescently labelled using Alexa 647 C2 maleimide (Thermo Scientific, Waltham, Massachusetts, USA) following the manufacturer’s instructions.

3.2.16 Electrophoretic mobility shift assay (EMSA)

1.5 µg of plasmid DNA containing linear, nicked and native forms in equal amounts was mixed in 20 µl of protein binding buffer (50mM NaCl, 20 mM Tris HCl pH7.5 and 0.2 mM EDTA) with varying amounts of rHMGB1 and incubated on ice for 40 min on ice. The samples were then loaded to a 1% agarose gel in 0.5x TBE and run for at 36 V for 15 hours at 4°C. The gel was stained with 0.5 µg/ml EBr and then imaged.

4. RESULTS

PART 1

4.1 EBr does not readily intercalate into DNA in native chromatin

Fluorescence microscopy revealed that when live cells are incubated with 10 μM EBr, the cells rapidly take up the dye and bright fluorescence is detected in the nucleoli and the cytoplasm. Minimal fluorescence was observed in the chromatin. The cytoplasmic fluorescence was likely due to dye intercalation into mitochondrial DNA and double-stranded RNA (dsRNA). The nucleolar signal was likely due to double-stranded ribosomal RNA as it was significantly diminished or eliminated when fixed nuclei were treated with RNase A. In permeabilized cells, there is observed chromatin staining, however the nucleoli stain more brightly.

To investigate the possibility that the low levels of chromatin fluorescence was due to a limited amount of dye permeating the cell membrane, we incubated live cells with 10 μM EBr and then in a whole-cell patch-clamp setup, directly microinjected a single cell with the same dye concentration. The patch-clamp apparatus used was coupled with a confocal microscope which allowed us to monitor staining in real-time. We observed that the dye gradually spread across the cytoplasm from the point of microinjection and into the nucleus where it stained the nucleoli first. EBr fluorescence in the chromatin was only observed after about 5 minutes and it took about 15 minutes before the chromatin was fully stained. During the whole observation period, the spatial distribution and level of EBr fluorescence in the neighbouring cells did not change and was much lower compared to that of the microinjected cell. The delay in staining indicated that, even when the dye concentration in the nuclei is sufficiently high, native chromatin resists intercalation. This differs substantially from mitochondrial DNA and dsRNA.

4.2 Intercalation into nucleosomal DNA closely correlates with nucleosome core particle disassembly

Early studies utilizing isolated chromatin fiber and reconstituted mononucleosomes had revealed that the nucleosome structure hampers intercalation into nucleosome-bound DNA. To investigate if the same applies to chromatin *in situ*, I developed an assay based on salt induced elution of

nucleosomes to determine the contribution of each of the histones to the inhibition. To this end, agarose embedded nuclei in the wells of an 8-well chamber were treated with various concentration of NaCl before being stained with EBr. I observed that the fluorescence of EBr remained unchanged for up to pre-treatment with 0.75 M NaCl. Above this concentration, a gradual increase in EBr staining was observed, up to 3-fold for samples treated with 1.55 M NaCl, i.e. a concentration at which all the histones have been eluted. The increase in EBr staining closely correlated with the elution of core histones which ensues at 0.75M NaCl for H2A-H2B dimers and 1.2 M NaCl H3-H4 tetramers. The results were identical for the HeLa cell line and for primary cells i.e. human peripheral blood lymphocytes. Similar observations were made with other intercalating dyes, psoralen and the bis-intercalator YOYO-1.

At the same time, there was an increase in the area of individual nuclei accompanying salt treatment. This increase begun at salt concentrations when the core nucleosomes were unaffected.

4.3 Initial intercalator binding is largely limited to the linker and nucleosome free regions

The findings above suggested that the initial binding of the intercalator was mainly limited to the nucleosome-free regions and internucleosomal DNA, and that intercalation into nucleosome bound DNA only occurred following nucleosome destabilization. To test this hypothesis, formaldehyde fixed nuclei were digested with MNase. This resulted in a spectacular decrease of EBr staining without a similar loss of mononucleosomes as deduced from the H2B-GFP fluorescence. This confirmed that the region initially stained was indeed linker DNA; sensitive to MNase digestion and hypersensitive to DNase I digestion.

Interestingly, the DAPI fluorescence, another small molecule that binds to the minor groove, was unaffected by the presence of nucleosomes either when applied alongside EBr or applied alone. This revealed that small molecules do indeed access nucleosomal DNA but intercalation (as in the case of EBr) is inhibited.

A previous study had suggested that EBr as well as its derivative PI bind both to DNA and histone proteins. To test whether such binding may have contributed to the increase in EBr fluorescence following histone elution, I carried out FLIM measurements on agarose embedded, RNA-depleted EBr stained nuclei. FLIM measurements detected only one lifetime component of 22.6 ns, which

corresponds to that of DNA-bound EBr. Further, through spectrofluorimetry measurement, it was found that a sufficient amount of the dye was left in the supernatant after staining, even in samples where the full histone compliment was present. This confirmed that, even if any EBr binding to histones occurred, it neither contributed to the fluorescence signal measured nor depleted the EBr which would reduce the dye available for intercalation.

Having ruled out limited access as well as binding of EBr by histones from among the possible mechanisms by which the nucleosome structure could inhibit intercalation, I investigated the possible role of the topological constraint exerted on the DNA by the nucleosomal structure. The DNA wrapped around the histone octamer is held in place by several hydrogen and electrostatic bonds which severely limit its degree of freedom. Removal of these bonds, as it occurs when histones are eluted by salt, may then increase the ability of DNA to deform to accommodate an intercalator. To assess the effect of constraint, I fixed salt pre-treated nuclei with either formaldehyde or ethanol then stained them with EBr and DAPI. In control experiments, the fixation was done post staining. The results showed that whereas ethanol had no effect on EBr intercalation, formaldehyde fixation negatively affected EBr intercalation. The effect of formaldehyde was much more pronounced in nuclei treated with 0.35 M NaCl when all the core histones were still present.

4.4 Enhancement of EBr intercalation by nicking of the DNA

Linker DNA is free of nucleosomes and its twist and writhe can freely interconvert however its ends are not free to rotate. To assess whether this would limit the level of intercalation, I first compared the EBr staining in plasmid DNA. Supercoiled DNA took up much less dye compared to the same amount of linear and nicked DNA indicating that the closed ends did indeed limit intercalation. Next I assessed whether nicking would equally increase dye intercalation *in situ*. Agarose embedded cell were irradiated with 300 Gy X-ray radiation, permeabilized and nuclei digested with RNase before being treated with varying concentrations of NaCl. I found that nuclei prepared from irradiated cells took up more EBr compared to control cells. The increase in EBr fluorescence of irradiated v.s. control samples is observed already <0.75 M NaCl, where intercalation is limited to the linker and NFR regions and the nucleosomes are in place. In other words, this increase was not due to more DNA becoming available for EBr intercalation as it would

happen when core histones are eluted, but due to NFRs taking up more dye. Above 0.75 M NaCl concentration, irradiation sensitized the core histones H2B-GFP to salt elution.

Transcription is expected to be an important determinant of the supercoiling state *in vivo*, so to check if it affects the binding of EBr *in situ*, I evaluated the binding of EBr to nuclei from transcription inhibited cells and compared it with the control. I observed a minimal increase in EBr binding in actinomycin D or α-amanitin treated cells.

The results above demonstrate that the intercalation of a small molecule to DNA *in situ* can be increased by relieving the topological strain imposed by packaging of DNA into chromatin. Salt-induced elution of histones, which perhaps mimics the nucleosome destabilization achieved by chromatin remodellers *in vivo*, converts the DNA around the NCP from a stiff toroid to a flexible plectoneme which is deformable, and whose twist and writhe can easily interconvert. Additionally, nicking of the DNA increased binding of the intercalator to the extranucleosomal DNA even in the presence of nucleosomes. Given these observations, I asked whether the binding of proteins with intercalating moieties to the extranucleosomal DNA would be similarly affected by alteration of DNA structure.

PART 2/A

4.5 Intercalating drugs, Dox and EBr, have a biphasic effect on HMGB1 binding *in vivo*

To evaluate the effect of changes in supercoiling and DNA helical properties on HMGB1 binding, live U2OS2FP cells were treated with varying concentrations of EBr and binding of HMGB1 was assessed by confocal microscopy. Uptake of EBr by the cells was evident from the appearance of red fluorescence in the cytoplasm and an increase in the red fluorescence in the nuclei. With increasing concentration of EBr, there was an observed loss of GFP-HMGB1 signal from the nucleoli, what was interpreted as either quenching of the GFP fluorescence by EBr intercalated in the dsRNA of ribosomal subunit or its recruitment to the chromatin. In the nucleoplasm, the GFP fluorescence signal appearance became more structured suggesting that the protein was becoming more chromatin bound. This hypothesis was confirmed by point FRAP measurements, in which the recovery time gradually increased from 25 ms for the control samples up to about 60 ms for

samples treated with 50 µM EBr. A further increase of EBr to 100 µM yielded a slight reduction in recovery time.

Similar to EBr, Dox caused GFP-HMGB1 loss from the nucleoli and a more structured distribution in the chromatin suggesting that the protein was becoming more chromatin bound. However, at Dox cc. > 9 µM, the GFP-HMGB1 signal again became diffuse suggesting that at higher cc. of Dox, the protein became more mobile. This was also confirmed by point FRAP, the recovery time increased with increasing Dox cc. peaking at 50 ms for samples treated with 4.5 µM and then declined with further increase in Dox cc.

Drug intercalation to DNA increases the base pair rise while reducing the helix twist by an angle dependent on the intercalator molecule. This decrease in helix twist translates to an overall reduction in DNA twist, which is compensated by an increase in writhe within the chromatin loops. To elucidate the contribution of superhelicity to the HMGB1 recovery profiles generated by intercalators, I carried out further experiments as outlined below.

4.6 DNA nicking had no effect on HMGB1 binding *in vivo*

To test the possible role of writhe in HMGB1 binding *in vivo*, DNA writhe was relaxed by exposing live U2OS^{2FP} cells to DNA nicking agents: H_2O_2, bleomycin or x-ray irradiation, and GFP-HMGB1 binding to DNA was evaluated by point FRAP. Given the short time interval between nicking and measurement, it was expected that the breaks would still be unrepaired and if they are at all repaired, the original levels of internucleosomal superhelicity, as established in S phase, would not have been regenerated. Thus, if HMGB1 binding is sensitive to negative writhe, its relaxation would reduce its binding. Surprisingly, none of these agents caused a change in the recovery rate.

To further test the effect of writhe, I evaluated the binding rHMGB1 to CCC plasmid DNA in the presence of varying cc. of Dox by FCS. First however, I evaluated the binding of fluorescently labelled rHMB1 to DNA in a gel retardation assay. The rHMGB1 was found to behave just as expected for the native protein which preferentially binds to supercoiled DNA. From the gel retardation assay, the migration of supercoiled plasmid DNA was retarded by as low as 945 ng of rHMGB1 (equivalent to 60:1 protein to plasmid molecules) and the retardation became more pronounced with increasing amounts of rHMGB1. Migration of the linear DNA was not affected

by up to 1575 ng of rHMGB1 (equivalent to 100:1 protein to plasmid molecules) and even then the retardation was only minute compared to supercoiled DNA. The nicked DNA band was not shifted at all for the protein concentration used in this experiment.

4.7 Dox decreases the binding of HMGB1 to supercoiled plasmid DNA

From FCS experiments, the diffusion coefficient of rHMGB1 in solution was found to be 86 $\mu m^2/s$. In the presence of DNA, I detected two components; a slow component diffusing at the rate of 5 $\mu m^2/s$ which was interpreted to be plasmid-bound HMGB1, and a fast component, 90 $\mu m^2/s$ which was inferred to be freely diffusing rHMGB1. Following addition of Dox, the fraction of the slow component was found to monotonously decrease from 35% in the absence of Dox to about 8% in the presence of 36 µM Dox. This observation suggests that Dox displaces or hinders binding of HMGB1 to DNA which could explain the decreased binding *in vivo* at concentrations of Dox > 4.5 µM.

4.8 Dox and EBr displace histone H1 from chromatin in live cells

HMGB1 competes with histone H1 for binding to the linker DNA. Further, daunomycin, a drug structurally similar to Dox was previously shown to evict the linker histone variant H1.1 from the chromatin. To test the involvement of H1-Dox interactions on chromatin in the HMGB1 recovery profiles observed *in vivo*, I treated HeLa cells expressing H1-GFP with Dox and analysed its effect my fluorescence microscopy. The effect of Dox on H1 was both time and dose dependent. Within 30 minutes, I observed relocation of H1 to the nucleoli, what was also reflected in decreased FRAP recovery time. At 120 minute, the nucleoli signal disappeared and there was a generalized loss of H1 from the cell. Similarly, EBr caused displacement of H1 from chromatin. These results suggest the increased binding of HMGB1 is due to loss of H1 which creates more binding sites.

PART 2/B

4.9 Translocation of HMGB1 to the nucleoli during fixation

In live U2OS, GFP-HMGB1 was distributed throughout the nucleus including the nucleoli. Following fixation with formaldehyde or glyoxal, I observed a chromatin to nucleoli translocation of HMGB1. This was hypothesized to be facilitated by the weak binding and fast diffusion which enables it to escape crosslinking to its sites on chromatin during the action of cross-linkers which

may take several minutes. Adding glutaraldehyde to formaldehyde significantly reduced this translocation, however it completely abolished IF labelling. IF labelling also failed to detect the HMGB1 accumulated in the nucleoli.

In ethanol fixed cells, HMGB1 was stripped from the chromatin and a small amount accumulated in the nucleoli. However, the nucleoli fraction was also lost in subsequent steps just as in Triton-X permeabilized cells.

DISCUSSION

PART 1

EBr intercalation into genomic DNA in live cells is generally thought to be solely inhibited by the cell membrane, and as such this dye has been used as a marker of cell viability. However, results presented herein reveal that a small amount of EBr does indeed traverse the cell membrane, apparently staining the mitochondrial DNA, and ds RNA in the ribosomal subunits present in the nucleoli. However, even when the dye is introduced into the cytoplasm and then enters the nuclei, as obvious from the ready staining of the nucleoli, its intercalation into nuclear DNA is still delayed. Similarly, Tramier and colleagues, observed no steady-state EBr fluorescence from the nuclei (nucleoli exempted) even though its presence could be detected through time correlated photon counting. These observations raise the possibility that there may exist stages in different forms of cell death when the membrane is permeable but the chromatin still resists intercalation.

Nucleosome impediment of intercalation was observed in an early experiment involving isolated chromatin fibres or mononucleosomes, however, whether this is manifested *in situ*, has not been studied before. To evaluate this, I developed an assay based on salt-induced destabilization of nucleosomes to sequentially elute histone proteins from agarose-embedded nuclei and assess their effect on EBr intercalation. Embedding cells into agarose prior to nuclei preparation ensures minimal perturbation to the chromatin. The assay had an RNase A digestion step to remove all DNA species as intercalators also bind to dsRNA. In agreement with those early findings, I found that the intercalation of EBr, YOYO-1 and psoralen into chromatinized DNA, at the conditions used, is limited to the linker regions and intercalation into nucleosomal DNA only occurs after nucleosome destabilization. The region that was initially stained by EBr in the presence of

nucleosomes was confirmed to be linker DNA as it was sensitive to MNase and hypersensitive to DNase I.

I have ruled out EBr binding to histones as a contributing factor to the shape of the EBr curve. EBr binding to histone proteins has been reported previously, even if such binding occurred in my experiments, there was still a large amount of dye left in the supernatant after staining the nuclei even in the presence of all the histones. Further, the dye fluorescence measured was confirmed to be that of DNA-bound EBr.

The fact that DAPI, another small molecule which binds to the DNA minor groove, was not affected by the presence of histones constitutes evidence against limited access as the means by which intercalation is inhibited. Thus, I hypothesized that intercalation, a step that follows access of EBr to DNA, is what is inhibited in chromatinized DNA. Inhibition is likely due to the tight grip of histones on DNA that greatly hinders its deformation to accommodate an intercalator.

During intercalation, a planar molecule is inserted between adjacent base pairs on a DNA molecule, which consequently affects the helix twist and rise to a degree dependent on the intercalator. YOYO-1 untwists the DNA by $\sim 24 \pm 8^{o}$ and increases the rise by 6.8 Å per molecule while EBr untwists the DNA by $\sim 26^{o}$ and increases the rise by 3.4 Å. Such DNA distortion would be disfavoured in a molecule of DNA that is constrained when wrapped around the NCP.

The 147 bp of DNA wrapped around the NCP is bound to the histone core by several direct and water mediated hydrogen bonds, van der Waals bonds and electrostatic bridges. Although histone-DNA interactions are highly dynamic, with the DNA spontaneously unwrapping from the nucleosomal ends, such events are transient and involve only a few base pairs (~ 10 bp). Thus, a large fraction of the nucleosomal DNA still remains constrained. This interpretation is in agreement with the finding that cross-linking the DNA to the nucleosome using formaldehyde further inhibits EBr intercalation unlike ethanol fixation which does not cause crosslinking and has no effect on EBr staining of nuclei. Following salt-induced eviction of histones, the constrained toroidal structures are converted to flexible plectonemes capable of altering their twist and writhe to accommodate intercalator molecules.

The increase in EBr binding following irradiation even in the presence of nucleosomes is in apparent support of the existence of unconstrained superhelicity. However, it could also be due to

the fact that in a nicked molecule, there is no build-up of positive writhe following intercalation. This means that even if linker DNA were initially relaxed, nicking would still increase intercalation. Even if this were the case, the increase in nuclear area observed for nuclei from irradiated cells beyond that of nuclei from control cells suggests that the chromatin loops are held in a supercoiled state, following DNA nicking, the loops relax and spread to occupy a larger area.

These evidence in support of the existence of net extranucleosomal torsion in the eukaryotic genome is at variance with some earlier experiments, in which gamma or X-ray radiation had no effect on psoralen photo-binding to genomic DNA in cells. This led to the conclusion that there is no net extranucleosomal torsion in eukaryotic cells. Recently however, Naughton and colleagues demonstrated a reduction in psoralen binding following treatment of cells with bleomycin, a DNA nicking agent. This discrepancy may arise from a number of factors: i) the results presented herein, as well as the immunofluorescence measurement referred to in Naughton *et al,* were obtained on a cell-by-cell basis as opposed to the population average in those early experiments, ii) the low concentration of psoralen used in the early experiments may have been a limiting factor, iii) immunofluorescence maybe more sensitive than quantification dsDNA resistant to denaturation or exonuclease digestion in the detection of intercalated psoralen. Upon UVA-illumination, psoralen forms just 1 crosslink for every 15 mono-adducts formed.

The global increase in EBr binding to the extranucleosomal regions was caused by a single nick per 50 kb chromatin loop. This implies that the effect of transient DNA breaks generated by topoisomerase spreads through the whole loop. However, it also suggests that persistent DNA breaks and superhelical domains must be somehow separated in the genome as both have been detected.

The increase in EBr binding to DNA following *in vivo* nicking implies that radiotherapy or chemotherapeutical agents that generate DNA breaks such as bleomycin and topoisomerase inhibitors may synergize anthracycline therapy.

In vivo, transcription is the main cause of superhelical changes. Following transcription inhibition by either actinomycin D or α-amanitin, EBr intercalation in the presence of nucleosomes was only slightly increased. This implies that at a genome-wide scale, transcription induces minimal net superhelical changes, hence the effect of transcription inhibitors is not detectable. This may be due

to symmetrical resolution of transcription induced torsional stress ahead and behind of the transcription machinery *in vivo*.

PART 2/A

Drug intercalation into DNA *in vivo* affects HMGB1 binding to DNA in a drug and concentration dependent manner. With increasing concentration, both EBr and Dox lead to recruitment and binding of HMGB1 to sites on chromatin reflected in both microscopy images and FRAP profiles. Above 9 µM Dox caused a drastic reduction in the binding of GFP-HMGB1 to DNA.

The initial increase in HMGB1 binding was found to be due to increased availability of binding sites following displacement of its competitor, histone H1, from linker DNA. Both Dox and EBr caused a dose-dependent displacement of histone H1 from chromatin.

The reduced HMGB1 binding to DNA above 4.5 µM was attributed to intercalator induced DNA distortion and/or competition for binding sites on DNA. Dox intercalation increases the base pair rise to 5.2 Å and reduces the helical twist at the site of intercalation. Untwisting of the DNA due to intercalation would be compensated for by positive writhing within the closed chromatin loops, in a dose dependent manner. This intercalator-induced change in torsion together with replication and transcription induced torsion is unlikely to be resolved by topoisomerases due to Dox induced inhibition of topoisomerases. Neither would the negative toroidal superhelicity resulting from histone eviction completely annihilate the positive torsion. The positive writhe would thus hinder the binding of HMGB1 because it involves intercalation. This argument is supported by the monotonous reduction in HMGB1 binding to covalently closed plasmid DNA in the presence of Dox.

In addition to intercalation of the anthraquinone moiety, the Dox amino sugar is positioned in the DNA minor groove. Similarly, HMGB1 binding to DNA involves both intercalation and minor groove binding. This overlap in the Dox and HMGB1 binding to DNA is bound to create grounds for competition and as the drug concentration is increased beyond 9 µM, HMGB1 is displaced from DNA. The Dox amino sugar which lies in the DNA minor groove has also been shown to sterically obstruct interactions between histones and DNA, and is responsible for histone displacement from DNA.

Even at 100 µM EBr, HMGB1 binding to DNA was markedly higher than that of the control. This is in a sharp contrast from the observation with Dox were at drug concentrations above 9 µM, markedly reduced its binding to DNA. This could be attributed to the lower uptake of EBr by live cells due to its positive charge, which means that the amount intercalated in DNA is much lower than that in the extracellular milieu. Doxorubicin on the other hand, is known to accumulate in the cells attaining higher intracellular concentration compared to the extracellular milieu. It may also be that the drastic reduction in GFP-HMGB1 binding at high Dox concentration is solely due to the presence of the amino sugar positioned in the DNA minor groove.

Despite the well documented preference of HMGB1 for supercoiled DNA *in vitro*, relaxing DNA by use of DNA nicking agents; X-ray irradiation, H_2O_2 or bleomycin, did not affect its binding *in vivo.* At the dosage used in these experiments, X-ray irradiation would cause 6,000 -50,000, while H_2O_2 would cause 15,000 -36,000 single strand breaks per nucleus. Despite the rapid repair of single strand breaks occurring within minutes, many of the breaks would still be unrepaired given the short time interval between nicking the DNA and measurement. Moreover, even if a large fraction were repaired it is unlikely that the level of supercoiling established in S-phase would have been restored within the short time. The fact that HMGB1 binding *in vivo* was not affected by DNA nicking means that the protein does bind to linear or relaxed DNA equally well when it is the only form available.

Nuclear to cytoplasmic translocation or extracellular secretion of HMGB1 from immune cells following ionizing radiation or H_2O_2 peroxide treatment, at doses lower than those used here, was observed by another group. This, however, occurred 3-24 hours after exposure, i.e. long after most of the single strand breaks would have been repaired and thus cannot be directly related to changes in superhelicity or H1 binding.

The evidence presented here suggests that supercoiling may not affect HMGB1 binding *in vivo*. To put it more accurately, any effect of supercoiling may be overshadowed by interactions involving histone H1.

The concentration dependent influence of Dox on HMGB1 binding has not been recognized before and may contribute to the effects and side-effects of this anthracycline of medical significance.

PART 2/B

Sample fixation and subsequent permeabilization are important steps in the immunofluorescence detection of intracellular components. Contrary to expectation, results presented here reveal that HMGB1 may escape crosslinking to its binding sites on chromatin and be subsequently relocated to the nucleoli. The escape is probably made possible by the weak binding of the protein to DNA, allowing it to detach and move to another location during the process of fixation which may take several minute. It is therefore important to always optimize fixation protocols to the component of interest. The observation also raises the intriguing question why nucleoli behave as depots of the protein released from chromatin.

CONCLUSION

The findings presented herein suggest that, the constraint of nucleosome-bound DNA imposed by the numerous histone-DNA bonds inhibits ligand binding to the DNA. An exception to this may be the pioneer TF which are able to bind DNA at the nucleosome entry/exit points during spontaneous unwrapping events or bind partial motifs and induce local release or distortion of DNA. Though the binding of the small molecule minor groove binder, DAPI, used in my experiments was unaffected by presence of nucleosomes, it may not be so for larger ligands that may require larger deformation. Further, due to the interrelated character of the DNA-shape features, the constraint imposed by the nucleosomes is expected to affect ligands with or without intercalating moieties that bind to DNA grooves or backbone. A number of TF that establish hydrogen bonds with DNA in the major groove also have moieties that extend to the minor groove.

On the other hand, the DNA devoid of nucleosomes encompassing linker DNA and NFR is flexible enough to allow deformation and ligand binding. For these regions, ligand binding appears to be strongly influenced, perhaps also regulated by competition between ligands.

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Registry number: DEENK/363/2021.PL
Subject: PhD Publication List

Candidate: Rosevalentine Bosire
Doctoral School: Doctoral School of Molecular Cell and Immune Biology

List of publications related to the dissertation

1. Zarębski, M.*, **Bosire, R.***, Wesołowska, J., Szelest, O., Eatmann, A., Jasińska-Konior, K., Kepp, O., Kroemer, G., Szabó, G., Dobrucki, J. W.: Translocation of chromatin proteins to nucleoli: the influence of protein dynamics on post-fixation localization.
   Cytom. Part A. [Epub ahead of print], 2021.
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   * These authors contributed equally this work.
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2. **Bosire, R.**, Nánási, P. P. i., Imre, L., Dienes, B., Szöőr, Á., Türk-Mázló, A., Kovács, A., Seidel, R., Vámosi, G., Szabó, G.: Intercalation of small molecules into DNA in chromatin is primarily controlled by superhelical constraint.
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   IF: 2.74

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List of other publications

3. Nánási, P. P. i., Imre, L., Firouzi Niaki, E., **Bosire, R.**, Mocsár, G., Türk-Mázló, A., Ausio, J., Szabó, G.: Doxorubicin induces large-scale and differential H2A and H2B redistribution in live cells.
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   DOI: http://dx.doi.org/10.1371/journal.pone.0231223
   IF: 2.74 (2019)

Total IF of journals (all publications): 8,604
Total IF of journals (publications related to the dissertation): 5,864

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

15 June, 2021