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Hydroxymethylation of DNA influences nucleosomal conformation and stability *in vitro*

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ABSTRACT

Background: Hydroxymethylation of DNA at the C5 position of cytosine (5hmC) is recognized as an important epigenetic mark. The molecular role of 5hmC in gene regulation, however, is not well understood.
Methods: We studied the effects of 5-hydroxymethylation (5hmC) on nucleosome properties in vitro using a combination of biochemical and fluorescence assays. Competitive reconstitution was used to evaluate the effect of 5hmC on nucleosome formation. The effects of 5hmC on nucleosome compactness and stability were characterized using FRET assays. These findings have also been compared with another important epigenetic mark, the cytosine methylation (5mC) of DNA.
Results: We observed that hydroxymethylation increases the binding affinity of DNA for the histone octamer. The formed nucleosome exhibits slightly different conformations based on the sequence and epigenetic context of DNA. DNA hydroxymethylation decreases the stability of formed nucleosomes in salt-induced dissociation processes.
Conclusion: DNA containing 5hmC is more likely to be incorporated into nucleosomes. Once formed, the 5hmC nucleosomes might be in an open and transcriptionally active state due to the weakened interaction of hydroxymethylated DNA with the H2A–H2B dimers.
General significance: Our results reveal the effect of 5hmC on regulating nucleosome compactness and stability in vitro.

1. Introduction

DNA methylation is a critical epigenetic modification that regulates a number of important biological processes such as stem cell differentiation, X-chromosome inactivation and the suppression of transposable elements [1,2]. It is a modification that primarily occurs in CpG sites and is associated with gene silencing and changes in chromatin structure [3,4]. The plasticity of the methylation state as well as its potential reversal and regulation pathways have been extensively studied in recent years [5–7]. It was found that the methylation state of DNA is dynamic and can be chemically modified by the Ten-Eleven Translocation (TET) proteins. TET proteins oxidize the methyl group on the cytosine and convert it to a hydroxymethyl group at the C5 position of cytosine (commonly known as 5hmC) [8,9].

5hmC is an oxidative product derived from 5mC and is a potential intermediary in the demethylation pathway [8]. The role of 5hmC in the demethylation process has been verified in the brain, zygotes and embryonic stem cells of mammals [10–12]. Accumulating evidence suggests that 5hmC, apart from its role in demethylation, serves as an epigenetic modification, which can directly regulate gene expression. For example, Mbd3 and Brg1 were found to regulate gene expression by having Mbd3 bound specifically to 5hmC and regulate the nucleosome occupancy of promoter regions [13].

The average 5hmC percentage across the genome is approximately 0.1–0.7%, which is about 10–100 times less than what is typically observed for 5mC [14,15]. 5hmC is enriched and conserved in important regulatory regions of genes, such as sequences near transcriptional starting sites, promoters, exons and insulator binding sites [16–18]. Specifically, in embryonic stem cells, certain CpG islands exhibit 5hmC levels of 3–24% in all CpG sites [14].

Epigenetic modifications regulate gene expression by altering the structure and functionality of chromatin. The nucleosome, consisting of 147 bp of DNA wrapped around a histone octamer, is the fundamental building block of chromatin [19]. *In vitro* studies have shown that DNA methylation can affect chromatin conformation by inducing changes in DNA rigidity [20], decreasing nucleosome-binding affinities [21] and enhancing DNA end "breathing" motion [22]. Histones also actively regulate the structure of nucleosomes [23,24]. Less is however known about the effect of 5hmC. Compared to 5mC, 5hmC includes an additional hydroxyl group to cytosine at the C5 position. This additional side chain can potentially increase the steric hindrance for nucleosome

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formation, while the polarity of the hydroxyl group may also facilitate nucleosome formation. There is no experimental evidence detailing the effects of 5hmC on chromatin conformation and activity.

This study aims to quantify the effects of 5hmC on nucleosome formation, conformation, and stability. We performed a side-by-side comparison of the effects of 5hmC and 5mC. Specifically, we introduced methylation and hydroxymethylation to three different types of DNA constructs, which differ on the basis of cytosine contents. The 5hmC percentage used in this study is higher than what typically occurs in cells. We decided to use a high overall 5hmC level, because it would allow us to unambiguously determine the overall effects of 5hmC. Additionally, the three constructs we used in this study contain additional cytosine patterns in the form of a $(CG)_5$ stretch and a $(CGX_8)_5$ repeat, the most frequently observed CpG patterns in the genome [25–27]. The relative differences in 5hmC levels among these three constructs are relatively small (<3%). Consequently, we will be able to assess how small changes in 5hmC levels impact the dynamic conformation of the nucleosome.

We characterized the ability of different DNA constructs to form nucleosomes using a competitive nucleosome reconstitution assay. The role of 5hmC in altering nucleosome stability and conformation was quantified using Förster Resonance Energy Transfer (FRET). Our results show that 5mC decreases the binding affinity of DNA for histone octamers, while 5hmC contrastingly increases the binding affinity. Methylation and hydroxymethylation can differentially affect the compactness of nucleosome, mainly through regulating DNA end breathing motions, depending on the sequence context. Both modifications act to decrease the stability of nucleosomes by potentially promoting dimer dissociation at increasing salt concentrations. The seemingly contrasting observations in nucleosome-forming ability and nucleosome-stability of 5hmC-containing DNA can be reconciled by the fact that 5hmC can decrease the binding affinities of DNA to H2A–H2B dimers, while increasing DNA affinities to H3–H4 tetramers.

2. Material and methods

2.1. Preparation of fluorescently labeled nucleosome samples

Three types of DNA constructs with different CpG patterns and cytosine densities were used in this study. The detailed DNA sequences and alignments are included in Table S1 and Fig. S1. The sequences of these DNA fragments were derived from the 157 bp Widom-601 sequence, which has one of the highest known binding affinities to histone octamers [28,29]. As DNA wraps around a nucleosome, it has contact points with the octamer at 10 bp intervals [19,30]. We therefore engineered two other sequences. These two additional sequences contain (i) an additional $(CG)_5$ stretch in the central dyad (later referred to as $(CG)_5$) and (ii) five CpG dinucleotide repeats at 10 bp interval (later referred to as $(CGX_8)_5$), where X stands for any type of nucleotides, respectively.

We used a PCR approach to incorporate methylated or hydroxymethylated cytosines in the DNA fragment. Specifically, instead of using normal dCTP in dNTP mixtures, we utilized the dNTP mixture containing methylated or hydroxymethylated dCTP (ZYMO Research). DNA fragments containing either methylated or hydroxymethylated cytosines were amplified using a label free DNA template and Taq DNA Polymerase (New England Biolabs). The DNA template was custom synthesized (Genscript). For each DNA construct, we prepared three types of samples, namely one control containing normal cytosines (control), one sample containing methylated cytosines (5mC) and another sample containing 5-hydroxymethylcytosines (5hmC). These DNA samples were fluorescently tagged using custom-made fluorescently labeled primers (Sigma).

For each DNA sample, we prepared three types of labeled DNA, (i) a donor-only sample tagged with fluorescein (FAM, donor), (ii) a dual-labeled sample tagged with FAM (donor) and tetramethylrhodamine (TAMRA, acceptor), and (iii) an acceptor-only sample tagged with TAMRA. The prepared DNA samples were analyzed using a 6% polyacrylamide gel and were purified using the same approach as we described previously [31]. A typical 6% gel of DNA samples is shown in Fig. S2(A) and (B). The labeling efficiencies of the samples were calculated by measuring the absorption spectra of the purified DNA fragments following an established approach [31]. The FAM and TAMRA labeling efficiencies were consistently observed to be >~90% and ~80%, respectively across all the constructs.

Mono-nucleosomes were reconstituted by mixing DNA constructs with refolded recombinant histone octamers at an optimized stoichiometric ratio as described before [31]. Labeled octamers used in alternative FRET studies were made by adding a FAM label to the serine 47 of H4 converted to cysteine as described before [31]. The reconstituted mono-nucleosome samples were examined using a 5% polyacrylamide gel as shown in Fig. S3. All samples exhibited a single translational setting and did not contain any unbound DNA fragments.

2.2. Time-domain fluorescence lifetime measurements

Förster Resonance Energy Transfer (FRET) was used to study the compactness of nucleosomes by evaluating the end-to-end distance of nucleosomal DNA as shown in Fig. 2 inset. The FRET efficiency was calculated based on the following equation:

$$E = 1 - \frac{\tau_{da}}{\tau_d} \quad (1)$$

where τ_{da} is the lifetime of the dual-labeled sample and τ_d is the lifetime of the donor-only sample. These fluorescence lifetimes were collected using a ChronosBH lifetime spectrometer (ISS) with similar settings as described previously [31]. All measurements were performed using samples prepared from >3 independent reconstitutions.

Anisotropy values of single labeled samples (with either FAM or TAMRA) were collected using a SpectraMax M5 Microplate Reader (Molecular Devices) at different salt concentrations ([KCl] ranging from 10–1150 mM). The collective anisotropy values are shown in Fig. S4. The anisotropy values are found to be consistently below 0.25 and show little dependence on salt concentrations or the type of cytosine modifications. The low anisotropy value suggests that the fluorescent labels can be considered freely rotating under the examined experimental conditions. Hindered rotation of dyes did not contribute to the observed changes in energy transfer efficiencies.

Ideally, the measured energy transfer efficiency values can be used to quantify the end-to-end distance of nucleosomal DNA. The TAMRA labeling efficiencies of DNA samples, however, were found to be less than 100% (~80%). Consequently, the energy transfer efficiencies will have contributions from the existence of a small fraction of donor-only molecules. Therefore, we will primarily use energy transfer efficiency (E) to assess the effects of 5hmC and 5mC on nucleosomal conformation and stability, while commenting briefly on the actual distance information. Higher E values correspond to a more compact nucleosome conformation.

2.3. Competitive nucleosome reconstitutions

The binding affinities of all DNA constructs to histone octamers were quantitatively examined using competitive nucleosome reconstitutions. The competitive reconstitutions were performed following an established protocol [32,33]. In the reconstitution, unlabeled Widom-601 sequence was used as the competitor DNA and mixed with the TAMRA labeled DNA of interest as well as the histone octamer. The competitor DNA concentration was kept at 3 μM, while the labeled DNA concentration was kept at 0.04 μM. We used a constant octamer concentration of 2.5 μM and kept the ratio of [octamer]:[DNA] as 0.85. This ratio was selected such that only a fraction of the labeled DNA was bound to the histone

octamer. The unmodified Widom-601 was used as a reference DNA to calculate the relative changes in binding energy as $\Delta\Delta G_i^{nuc}$ (kcal/mol).

Reconstituted samples were then analyzed on a 5% native polyacrylamide gel and imaged using a Kodak 4000R Imaging Station (Kodak). The intensities of the labeled DNA bands either as free DNA or as nucleosomes were quantified using ImageJ. The relative change in binding energy ($\Delta\Delta G_i^{nuc}$) was calculated following Eq. (2), similar as described by Thåström et al. [33].

$$\Delta\Delta G_i^{nuc} = -RT \ln\left(\frac{K_i}{K_{ref}}\right) \quad (2)$$

where, $K_i = Intensity_{NCP}/Intensity_{DNA}$ and the reference is the Widom 601 unmodified DNA. Higher $\Delta\Delta G_i^{nuc}$ values indicate lower binding affinities.

3. Results

3.1. DNA modifications affect the histone octamer-binding affinity of DNA

We started by evaluating the binding affinities of various DNA constructs to histone octamers. The binding affinities of DNA to histone octamers account for their likelihood of being incorporated into nucleosomes and ultimately regulate chromatin structure by determining the nucleosome-positioning pattern of chromatin [32,34]. We performed competitive nucleosome reconstitutions to measure the free energy difference for nucleosome formation ($\Delta\Delta G_i^{nuc}$) based on an established protocol [32,33]. Lower $\Delta\Delta G_i^{nuc}$ values indicate a higher binding affinity and a higher probability of being occupied by nucleosomes.

A typical gel analyzing the products from competitive nucleosome reconstitution is shown in Fig. 1(A). The unmodified Widom-601 sequence was used as the competitive sequence and also reference DNA in our assay. At a selected DNA to octamer ratio (0.85), unmodified and 5hmC-containing DNA fragments both form a significant amount of nucleosomes. No nucleosome formation, however, was observed for DNA fragments containing 5mC. This observation suggests that DNA methylation significantly reduces the binding affinities of DNA to histone octamers consistent with previous reports [35,36].

Using the same DNA to octamer ratio, both unmodified and hydroxymethylated DNA can be incorporated into nucleosomes. We calculated $\Delta\Delta G_i^{nuc}$ of these DNA fragments and the results are summarized in Fig. 1(B). Unmodified Widom-601 sequence was used as reference DNA and thus by definition has $\Delta\Delta G_i^{nuc}$ equal to 0. Altering the sequences of DNA does not significantly change its likelihood of forming nucleosomes. The unmodified Widom-601 sequence exhibits the highest binding affinity among all unmodified constructs, consistent with literature reports [28]. Converting cytosines to 5hmC significantly reduces $\Delta\Delta G_i^{nuc}$ of the corresponding DNA fragments. The changes are most significant for the DNA fragment containing additional CG dinucleotide repeats, suggesting that the addition of 5hmC at 10 bp intervals (the typical helical repeat length of nucleosomal DNA [30]) can preferentially facilitate nucleosome formation.

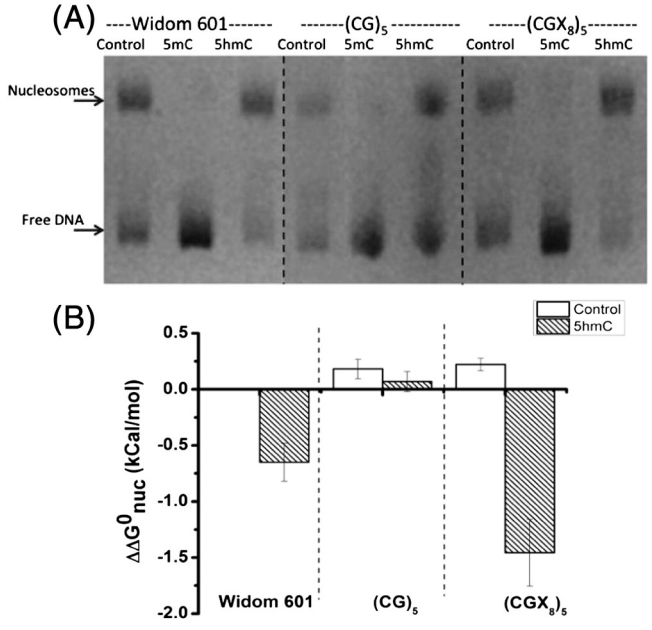

Fig. 1. (A) Typical 5% polyacrylamide gel analyzing the competitive reconstitution results. The unlabeled competitor DNA used in this assay was the Widom 601 sequence at a concentration of 3 μM. The labeled DNA concentration was 0.04 μM and the histone octamer concentration was 2.5 μM. The [octamer]:[DNA] was 0.85. Three different sets of reconstitutions were carried out for each of the three constructs. (B) Graph showing the $\Delta\Delta G_i^0$ values obtained from >3 independent reconstitutions ($p < 0.005$ while comparing 5hmC and control samples). Control: Widom 601 DNA.

3.2. DNA methylation and hydroxymethylation affect nucleosome compactness in a sequence dependent manner

Once nucleosomes have been assembled, they continue to undergo conformational changes depending on salt concentrations *in vitro*, which is a proxy for accessibility modulation *in vivo* [37–41]. It has been shown that at low salt concentrations, i.e., [KCl] <200 mM, nucleosomes assume a fairly stable conformation and DNA-end breathing motion is the primary dynamic feature of the protein–DNA complex [31,37,42–44]. This end breathing motion is thought to control accessibility of DNA. We thus examined the dynamic conformation of nucleosomes under two salt concentrations, namely [KCl] = 10 mM and 100 mM, close to the physiological condition.

A FRET pair (FAM/TAMRA) was introduced to the 5′ ends of nucleosomal DNA as shown in Fig. 2 inset. The measured energy transfer efficiencies (E), therefore, characterize the relative compactness of formed nucleosomes. The results obtained at 10 and 100 mM [KCl] are illustrated in Figs. S5 and 2, respectively. We first examined the sequence dependence of the measured *E*. The Widom-601, $(CG)_5$ and $(CGX_8)_5$ constructs exhibit decreasing values of *E*, indicating more open conformations. The difference is most pronounced between Widom-601 and $(CGX_8)_5$ constructs. These results mirror our previous findings [22]

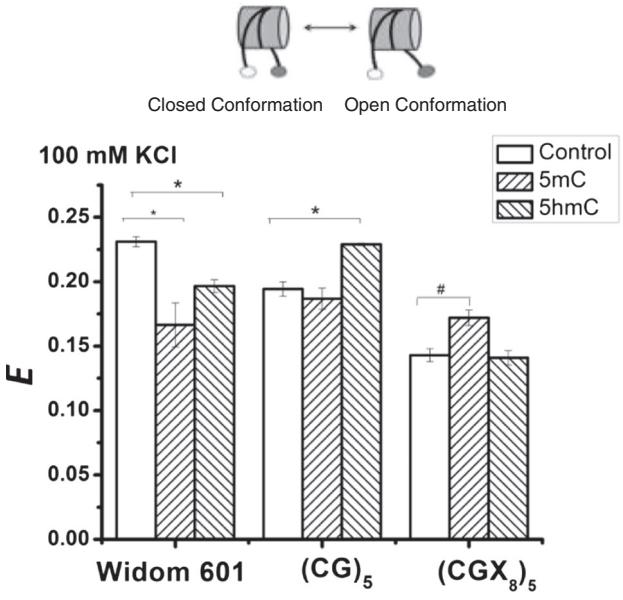

Fig. 2. Energy transfer efficiencies of reconstituted mono-nucleosomes at KCl = 100 mM. The inset illustrates the labeling position of the FRET pair and the two possible nucleosomal conformations, closed and open conformations. Data = mean ± standard error (n > 3) and *: *p* value < 0.0001, #: *p* value < 0.001.

and also match sequence based nucleosome occupancy predictions using a web-based tool [45]. This consistency potentially suggests that the nucleosome occupancy map can be partially determined by the TA, TT, AA and GC dinucleotide phasing as revealed in previous literature [45].

Increasing salt concentration from 10 to 100 mM slightly increases E by <10%, suggesting that a more compact conformation is being adopted, which can be attributed to the neutralization of surface charges of DNA by K^+ cations.

The effects of both 5mC and 5hmC, interestingly, exhibit significant dependence on the DNA sequence context. Specifically, methylation of all cytosines leads to a decrease in E values for the Widom-601 sequence ($p < 0.0001$), Fig. 2. The 5mC DNA constructs with additional $(CG)_5$ did not have significantly different E values from the unmodified controls. However, a pronounced increase in E was observed in nucleosomes containing the additional $(CGX_8)_5$, suggesting a more compact conformation due to cytosine methylation ($p < 0.001$). 5hmC leads to a decrease in the E value for the Widom 601 sequence ($p < 0.0002$), while causing a significant increase in the energy transfer efficiency for the $(CG)_5$ construct ($p < 0.0001$). Almost no change in E was seen for the $(CGX_8)_5$ construct due to hydroxymethylation. Relative changes in E values caused by 5mC and 5hmC are plotted in Fig. S6(A) and (B).

The relative change in E values (wherever significant difference was observed) translates to a distance change of ~0.2–0.4 nm (estimated using $R_0 = 5$ nm). The distance change suggests that around 2–4 bp of DNA (0.34 nm/bp) at the nucleosome entry/exit sites can be affected by the DNA sequences and modifications. Since the methylation and hydroxymethylation levels that we introduced to DNA are much higher than what typically occurs in living cells, we do not expect DNA modifications to significantly alter the static conformation of chromatin *in vivo*. The changes in dynamic features, e.g., changes in DNA end breathing motions induced by hydroxymethylation, may have an effect on the initiation of transcription events, such as when a transcription factor or a remodeling factor is trying to access a specific DNA sequence. Overall, 5mC and 5hmC might affect nucleosome conformation in certain DNA sequence context.

3.3. DNA methylation and hydroxymethylation reduce nucleosomal stability

The nucleosomal stability partially encodes for the accessibility of DNA to various nuclear proteins and may determine the transcription state of chromatin [34,46]. DNA epigenetic modifications and histone post-translational modifications are both known to contribute to the stability of nucleosome [22,37,39,47,48]. We measured the E values of labeled mono-nucleosomes at increasing salt concentrations (0–1200 mM KCl), with typical results shown in Fig. S7. The salt concentration at which half the FRET signal was lost (termed as C_{50}) was used to characterize the stability of mono-nucleosomes. Lower C_{50} values suggest a less stable nucleosome and similar approaches have been widely adopted to monitor nucleosomal stability and dissociation pathways in literature [22,37,38].

Nucleosomal stability does not vary significantly for the three different sequence constructs in the unmodified state, as seen in Fig. 3(A). The most pronounced changes in the C_{50} values arise from the addition of methyl and hydroxymethyl cytosines. Independent of the sequence context, these modifications decrease the C_{50} value and thus the stability of nucleosomes ($p < 0.001$). The relative change induced by both modifications, calculated as $(C_{50,(5mC/5hmC)} - C_{50,control}) / C_{50,control}$, where $C_{50,(5mC/5hmC)}$ and $C_{50,control}$ is the C_{50} value of nucleosomes containing 5mC or 5hmC and unmodified control, respectively were not significantly different from each other, as seen in Fig. 3(B) (tested with $p = 0.5$).

However, given the enhanced affinity of 5hmC DNA for the histone octamer, the reduced stability values of the 5hmC nucleosome (as compared to the unmodified sequence) were a surprising finding. We resolved this apparent contradiction by examining the affinity of 5hmC to H2A–H2B dimers. An alternative labeling strategy (DNA end (acceptor)) and a histone octamer (donor located at H4) were also used to examine the nucleosome dissociation process. Nucleosome dissociation typically starts with dimer dissociation and ends with the DNA peeling off from H3–H4 tetramers. If 5hmC DNA has a reduced affinity for the dimer alone, the C_{50} values measured using the alternative FRET strategy will be similar, while 5hmC should show lower affinities to H2A–H2B dimers. This hypothesis was tested by competitive binding assays conducted using the unmodified DNA, 5hmC DNA and the (H2A–H2B) dimer. The results are presented in Figs. 4A and S8A. The $\Delta\Delta G_i^{nuc}$ values of 5hmC DNA and dimers are higher when compared to the unmodified sequence ($p < 0.0003$), suggesting a reduced affinity for H2A–H2B dimers. Similar trend was observed for the $(CG)_5$ and $(CGX_8)_5$ constructs as shown in Fig. S9.

Using the alternative FRET labeling strategy, we examined the FRET signal between the octamer surface and the DNA end over a salt gradient of 0–2 M KCl. By choosing this labeling location, we were able to

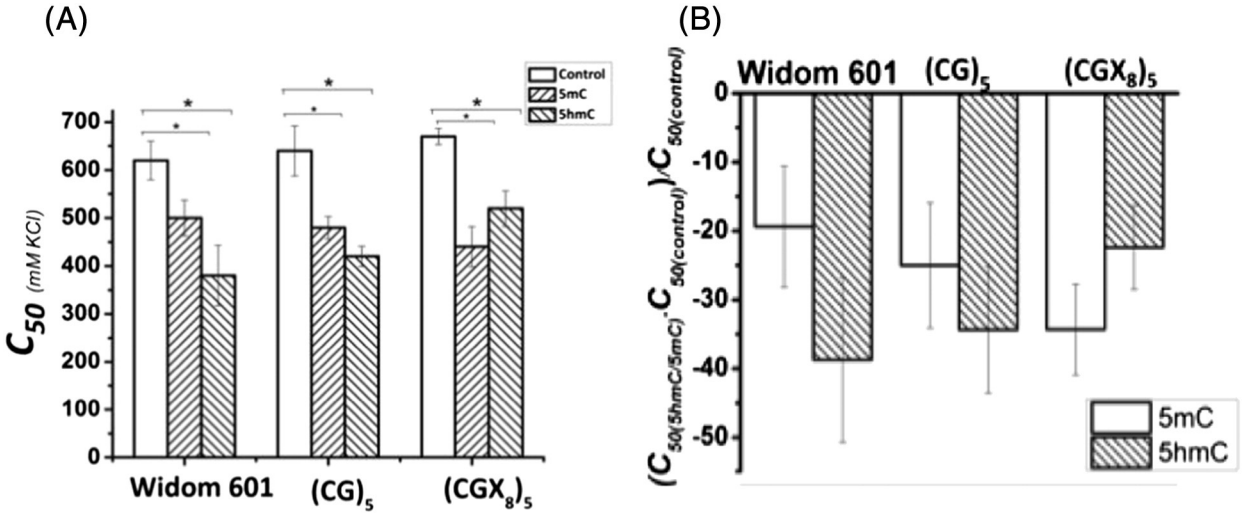

Fig. 3. (A) A summary of C_{50} values of all DNA constructs with different DNA modifications. (B) The relative changes in C_{50} values due to cytosine modifications. There is no significant difference between the effects of 5mC and 5hmC as compared to the unmodified constructs (n > 3). These values were obtained from the results of three (or more) independent sets of reconstitutions for all three constructs.

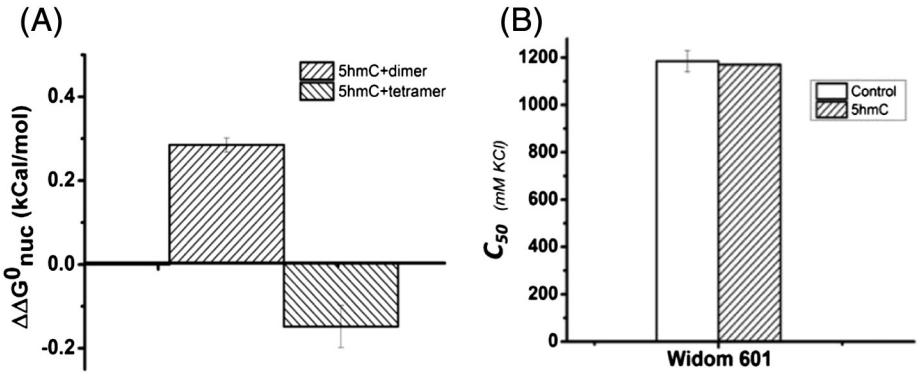

Fig. 4. (A) Graph showing the $\Delta\Delta G_i^{nuc}$ values obtained from 4 independent reconstitutions of unmodified DNA, 5hmC DNA, dimer or tetramer. ($p < 0.0003$ while comparing 5hmC and unmodified Widom-601 for the dimer and $p < 0.05$ for the tetramer). Control: Widom 601 DNA. (B) The C_{50} values of unmodified or hydroxymethylated nucleosomes measured using the alternative FRET labeling strategy. There is no significant difference between the 5hmC and the unmodified DNA ($n > 3$, $p < 0.6$). These values were obtained from the results of three (or more) independent sets of reconstitutions.

capture the nucleosomal dissociation dynamics governed by DNA and histone tetramer interactions. The dissociation curves are presented in Fig. S10. We found that C_{50} values were not statistically different between the unmodified and the 5hmC nucleosomes, as shown in Fig. 4B. These results were further supported by binding assays conducted using (H3–H4) tetramers. Our results, as shown in Figs. 4A and S8B, suggest that the $\Delta\Delta G_i^{nuc}$ values were lower in the case of the 5hmC DNA and tetramer ($p < 0.05$) compared with unmodified DNA and tetramer. Overall, our results confirmed that 5hmC as a modification reduces the affinity of the DNA for the histone dimer (H2A–H2B). The same modification shows an increased affinity for the histone tetramer (H3–H4).

4. Discussion

The histone octamer-binding affinities, conformation and stability exhibit different dependence on the DNA sequence and presence of DNA modifications. These variations are somewhat expected, since they reflect different dynamic features of nucleosomes. Specifically, the measured nucleosome conformation is expected to be determined by the DNA end breathing motions. The nucleosomal stability monitored in our study has been proposed to primarily capture the dimer destabilization process at modestly high salt concentrations [37,38], while the binding affinities of DNA to histone octamers include contributions from physical properties of the DNA sequence and their interactions with positively charged histone octamer surfaces.

The nucleosome-binding affinity quantifies the likelihood of individual DNA sequences being occupied by histone octamers [34]. The sequence dependence in the $\Delta\Delta G_i^{nuc}$ values of unmodified DNA sequences is likely to originate from various structural properties of individual sequences (such as twisting and bending) as suggested in the literature [33,49]. Methylated DNA shows reduced binding affinity for the histone octamers, similar to what has been observed in previous literature [35,50] using DNA containing cytosine methylation within CG dinucleotides. This reduction has been attributed to the hydrophobicity of the methyl group [36]. Interestingly, the more hydrophilic 5hmC showed a trend opposite to 5mC by increasing the binding affinity of the DNA for the tetramer. This finding suggests that the additional hydroxyl group in 5hmC would compensate for the hydrophobicity of methyl group. Additionally, the hydroxyl groups may also facilitate nucleosome formation via additional hydrogen bonds.

The DNA end-breathing motion can contribute to the accessibility of nucleosomes to transcription factors. This motion can be primarily attributed to the physical properties of DNA fragments entering and exiting from nucleosomes. Methylated DNA exhibits higher rigidity [51–53] and consequently leads to enhanced end breathing and a more open conformation in nucleosomes as demonstrated in the Widom 601 and $(CG)_5$ constructs. However, the methylation at $(CGX_8)_5$ position causes a compaction effect. This location corresponds to additional cytosines in direct contact with the histone octamer. Methyl side chain at this location would face away from the histone octamer and into the broadened major groove. These additional contacts between methylated cytosines and histone octamers seem to help stabilize nucleosome conformations. Similar observations have been found in genome-wide association studies [26], suggesting that methylated $(CGX_8)_5$ may impact DNA–histone octamer interactions or DNA geometry.

Introduction of 5hmC to the Widom 601 ($p < 0.0002$) and the $(CG)_5$ ($p < 0.0001$) constructs leads to a more compact conformation as compared to the methylated counterparts. These effects can be attributed to the increased flexibility imparted to the DNA by hydroxymethylation, which has been reported previously [52]. The hydroxymethylated $(CGX_8)_5$ construct, however, shows decreased E value compared to the methylated counterpart ($p < 0.001$), implying an increased end-to-end distance. This finding suggests that hydroxymethylated cytosines may interact with histone octamers in a different way from methylated cytosines.

The nucleosome stability, as measured here for all three constructs using labels on the DNA ends, accounts for the process of dimer destabilization and progressive unwrapping of DNA [22,37,45]. Although it was expected that nucleosomes that form more readily should also demonstrate higher stability during the dissociation experiment, our results were not in exact accordance to this expectation. The C_{50} values obtained for both 5mC and 5hmC constructs show a decrease in nucleosomal stability compared to the unmodified DNA.

Interestingly, our results for 5hmC show that while it increases the binding affinity of DNA for the octamer, it also causes a decrease in stability of the formed nucleosomes. A similar observation has been noted in a previous study comparing the stability and conformations of nucleosomes containing 5S NPS and Widom-601 sequences. This study suggests that higher $\Delta\Delta G^{nuc}$, but lower site exposure equilibrium of 5S NPS as compared to the 601 sequence arises from the higher affinity of the 5S NPS to the H2A–H2B dimer than the Widom 601 sequence [32,54]. The discrepancy observed in our experiments can also be addressed by the fact that the stability measurements were carried out using FRET pairs attached to nucleosomal DNA ends. Consequently, C_{50} values as we obtained in this study will primarily reflect the dimer stability of nucleosomes, i.e., the first step of nucleosome dissociation pathway as suggested in the recent mechanical study of mono-nucleosomes [37]. Noting the results in previous literature [32,54] and combining it with the dimer stability values obtained by ourselves, this contradiction can be explained by the fact that 5hmC DNA has a lower affinity for the H2A–H2B dimer, but a higher affinity for the H3–H4 tetramer. Hydroxymethylated DNA could thus be more transcriptionally accessible, consistent with its proposed biological role as a demethylation intermediate to reverse transcriptional silencing events [5,14].

In conclusion, our findings show that hydroxymethylation of cytosines can have a significant impact on nucleosomal properties. Hydroxymethylation leads to increased histone octamer-binding affinities and is more likely to be incorporated into nucleosomes. Once formed, hydroxymethylated nucleosomes demonstrate dynamic nucleosome conformations depending on the underlying DNA sequence contexts. Surprisingly, 5hmC decreases nucleosomal stability due to weak interactions between DNA and H2A–H2B dimers. Compared with methylation, hydroxymethylation plays an opposing role in nucleosome assembly but demonstrates similar destabilization effect on assembled nucleosomes. This property is consistent with the biological functionality of DNA hydroxymethylation. This modification is abundant in regions such as TSS, promoters and exons of actively transcribed genes [14,55]. Our findings reveal the effects of 5hmC on nucleosomal properties, which have never been studied before to the best of our knowledge. It paves the way towards understanding how specific DNA modifications regulate transcriptional dynamics in the context of CpG sites.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagrm.2014.09.014.

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