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The chromatin-binding protein HMGN3 stimulates histone acetylation and transcription across the *Glyt1* gene

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Abstract

HMGNs are nucleosome-binding proteins that alter the pattern of histone modifications and modulate the binding of linker histones to chromatin. The HMGN3 family member exists as two splice forms, HMGN3a which is full-length and HMGN3b which lacks the C-terminal RD (regulatory domain). In the present study, we have used the *Glyt1* (glycine transporter 1) gene as a model system to investigate where HMGN proteins are bound across the locus *in vivo*, and to study how the two HMGN3 splice variants affect histone modifications and gene expression. We demonstrate that HMGN1, HMGN2, HMGN3a and HMGN3b are bound across the *Glyt1* gene locus and surrounding regions, and are not enriched more highly at the promoter or putative enhancer. We conclude that the peaks of H3K4me3 (trimethylated Lys⁴ of histone H3) and H3K9ac (acetylated Lys⁹ of histone H3) at the active *Glyt1a* promoter do not play a major role in recruiting HMGN proteins. HMGN3a/b binding leads to increased H3K14 (Lys¹⁴ of histone H3) acetylation and stimulates *Glyt1a* expression, but does not alter the levels of H3K4me3 or H3K9ac enrichment. Acetylation assays show that HMGN3a stimulates the ability of PCAF [p300/CREB (cAMP-response-element-binding protein)-binding protein-associated factor] to acetylate nucleosomal H3 *in vitro*, whereas HMGN3b does not. We propose a model where HMGN3a/b-stimulated H3K14 acetylation across the bodies of large genes such as *Glyt1* can lead to more efficient transcription elongation and increased mRNA production.

Keywords

acetylation; chromatin; elongation; epigenetics; HMGN; p300/CREB (cAMP-response-element-binding protein)-binding protein-associated factor (PCAF)

INTRODUCTION

HMGN (high-mobility group nucleosome-binding) family members are nuclear proteins that bind to nucleosomes and alter chromatin structure and function (reviewed in [1]). They modulate transcription and thus regulate gene expression patterns *in vivo* [2,3], as well as being important for DNA repair [4] and replication [5]. Studies using knockout mice and

cultured cells have revealed roles for HMGNs in early embryogenesis, in differentiation and in the response to various stresses (reviewed in [6]). There are four canonical members of the family, HMGN1–4, and they share a highly conserved NBD (nucleosome-binding domain), a bipartite NLS (nuclear localization sequence/signal) 1 and 2, and an RD (regulatory domain) [7]. A related protein named HMGN5 also contains the conserved NBD, but has a large C-terminal acidic RD [8]. In the present study, we focus on the role of HMGN3, which is the only HMGN family member to exist as two splice variants, HMGN3a and HMGN3b [3,9]. The shorter HMGN3b variant lacks the C-terminal RD, but it has not been shown whether the two variants play distinct roles *in vivo*.

Many studies have investigated the mechanisms used by HMGNs to influence transcription. Experiments using chromatin templates assembled *in vitro* revealed a role for HMGNs in unfolding chromatin and modulating transcription [1,10–12]. Both *in vitro* and *in vivo* studies have demonstrated that HMGNs can alter chromatin structure in a variety of ways, including counteracting linker histone H1 [13], inhibiting chromatin remodelling complexes [14] and altering the level of histone modifications. In particular, HMGN2, and to a lesser extent HMGN1, stimulate acetylation of nucleosomal H3K14 (Lys¹⁴ of histone H3) by PCAF [p300/CREB (cAMP-response-element-binding protein)-binding protein-associated factor] *in vitro* [15,16]. HMGNs can also modulate the MSK1 (mitogen and stress-activated kinase 1)- and RSK2 (ribosomal S6 kinase 2)-mediated phosphorylation of H2AS1 (Ser¹ of histone H2A), H3S10 (Ser¹⁰ of histone H3) and H3S28 (Ser²⁸ of histone H3) in nucleosomal substrates [16–19]. Analyses of domain-swap and deletion mutations have revealed that the RD of HMGN2 is responsible for stimulating H3K14 acetylation by PCAF, whereas the NLS2 region of HMGN1 is responsible for inhibiting H3S10 phosphorylation by MSK1 [16].

There are several reports of functional and/or physical interactions between HMGNs and transcription factors, including TR/RXR (retinoid X receptor) [20], PITX2 (pituitary homeobox 2)-β-catenin [21], ERα (oestrogen receptor α) [22], SRF (serum-response factor) [22] and PDX-1 (pancreatic and duodenal homeobox-1) [23]. In most of these cases, the transcription factor is responsive to extracellular signals [e.g. thyroid hormone (TR/RXR), oestrogen (ERα and SRF) and Wnt signalling (PITX2-β-catenin)]. The extracellular signal thus acts via the transcription factor to regulate HMGN binding [21,22]. Furthermore, in the examples of TR/RXR, PITX2-β-catenin and PDX-1, the HMGN protein also seems to promote the DNA binding of the transcription factor [20,21,23]. Thus HMGNs could influence transcription by modulating the DNA binding of specific transcription factors, and in some cases this appears to be independent of the effects of HMGNs on chromatin structure [20,21].

The ability of HMGNs to affect transcription will also depend on where HMGNs are bound with respect to individual genes. Few studies have combined functional analysis of how HMGNs regulate a gene with detailed analysis of where HMGNs bind to the gene in question. In the examples mentioned above, HMGN binding at a gene promoter is stimulated by certain transcription factors, and results in either activation or repression of the gene. In the case of *Sox9*, however, HMGN1 is enriched at other regions across the locus rather than the promoter, and is thought to repress gene expression in mouse limb bud cells [24]. At the *Hsp70* (heat-shock protein 70) gene, HMGN1 is bound evenly across the entire gene locus but promotes histone acetylation at the nucleosome near the promoter, and stimulates heat-shock-induced transcription at early time points [25]. A recent genome-wide study by Zhao and co-workers found that HMGN1 is preferentially bound to the promoters of active genes and at DHSs (DNase I-hypersensitive sites) [26]. However, there is not yet any functional data to show whether all of these genes are actually regulated by HMGN1.

In the present study, we focused on a known HMGN3 target gene, *Glyt1* (glycine transporter 1, also known as *Slc6a9*) [3] to investigate where the two HMGN3 splice variants bind to this locus and how they modulate gene expression. *Glyt1* plays an essential role at glycinergic and glutamatergic synapses in the brain and CNS (central nervous system) (reviewed in [27]), but is also expressed in several other tissues including the liver, lung and pancreas [28]. We have previously shown that *Glyt1* is up-regulated by overexpression of HMGN3 in murine Hepa-1 cells [3], and we wanted to investigate which regions of the gene are preferentially bound by HMGN3a/b. We were particularly interested in whether modifications of the histone H3 N-terminal tail might play a role in HMGN3 targeting, as there is evidence that HMGNs interact with the N-terminal tail of histone H3, and that the presence of histone tails stabilizes the interaction of HMGNs with nucleosome core particles [29,30]. We also aimed to investigate whether the binding of either full-length HMGN3a or the shorter HMGN3b alters the pattern of histone modifications at *Glyt1* and how this might be related to the activation of gene expression.

EXPERIMENTAL

Plasmid construction

Insulator sequences from plasmid pBAW3 [a gift from G. Felsenfeld, NIH (National Institutes of Health)] were inserted on either side of the expression cassette of plasmid pTRE (Clontech) to minimize chromatin position effects after random integration into the genome. Tandem copies of the core insulator from HS4 of the chicken β -globin locus were digested with BglII/SpeI, blunt-ended and either had XhoI linkers ligated for insertion into the XhoI site or were ligated into the blunted SapI site of pTRE to create pTRE-INS2. Both insulator insertions are in the same direction. The open reading frame of mouse HMGN3a or HMGN3b was amplified by PCR and inserted into the MluI and SalI sites of pTRE-INS2 to create pTRE-INS2-N3a and pTREINS2-N3b.

Generation and culture of HMGN-expressing cell lines

The 'control' cell line (clone 2–9) was derived from the mouse hepatoma line Hepa-1. Cells were transfected with pTet-ON and pTet-tTS (Clontech) and selected in DMEM (Dulbecco's modified Eagle's medium) with 10% FBS (fetal bovine serum) and 400 μ g/ml G418. H-N3b (clone 293-73) and H-N3a (clone 291-10) cell lines were derived from clone 2–9 following transfection with pTK-hyg and either pTRE-INS-N3b or pTRE-INS2-N3a respectively, and selection with 400 μ g/ml hygromycin. For HDAC (histone deacetylase) inhibitor treatments, control cells were incubated with 2 mM or 5 mM sodium butyrate, or 165 nM trichostatin A for 3 h. These concentration ranges have been used previously to inhibit deacetylases and induce gene expression in Hepa-1 and human hepatoma cell lines [31–33].

RT (reverse transcription)–PCR

RNA was extracted using RNeasy kits (Qiagen), and reverse transcribed using Superscript III reverse transcriptase (Invitrogen) and oligo(dT)₁₆ according to the manufacturer's instructions. An aliquot of the cDNA was used in real-time PCR using SYBR Green (Roche) using a Stratagene Mx3000P Q-PCR machine according to the manufacturer's instructions. The PCR primer sequences are listed in the Supplementary Tables S1 and S2 (available at <http://www.BiochemJ.org/bj/442/bj4420495add.htm>). Primer sequences for *Gcn5* (general control of amino acid synthesis 5), *Pcaf* and *p300* are courtesy of OriGene. For each sample, the mean Ct from three replicates was taken. Expression levels were normalized to *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase) and the control sample using the comparative $\Delta\Delta C_T$ method.

ChIP (chromatin immunoprecipitation) and Western blotting

ChIP was carried out as described by Duan et al. [34] with minor modifications. This protocol results in significantly higher immunoprecipitation efficiency and reproducibility for HMGN3 ChIPs, compared with that described previously [3]. Cells were removed from culture plates by scraping into growth medium. Protein-DNA cross-linking was performed by incubating cell suspensions with formaldehyde at a final concentration of 0.5% for 5 min at room temperature (23 °C) with gentle agitation. Glycine was added to 0.125 M and incubated for 5 min to quench the reaction. Chromatin was sonicated to an average size of 500 bp. Aliquots were pre-cleared with normal IgG for 1 h followed by incubation with Protein G-agarose (Millipore) overnight. Pre-cleared chromatin was then incubated with 4–10 µg of specific antibody for 2 h at 4 °C. Normal IgG (10 µg) control immunoprecipitations were always performed in parallel to experimental IPs for each batch of chromatin. Immune complexes were collected by incubation with 30 µl of Protein G-agarose for 2 h at 4 °C. Complexes were washed and eluted, cross-links were reversed, and DNA was purified by Qiagen MinElute columns. For Western blotting, cross-links in chromatin samples were reversed by heating to 95 °C for 20 min in SDS/PAGE loading buffer. Perchloric acid extracts for HMGN3 Western blots were prepared as described previously [35] and concentrations assayed by measuring the A_{280} using an ND1000 Nanodrop spectrophotometer. Samples were run on SDS/15% PAGE gels and blotted on to PVDF, and membranes were probed with the antibodies listed above. Blots were imaged using a Fujifilm LAS3000 imager and quantified using Aida Image Data Analyzer software.

Antibodies

The N3-Cter antibody was raised in rabbit against the peptide VEEAQRTEISIEKEGE and affinity purified (Eurogentec). It is specific to HMGN3, and does not recognize HMGN1 or HMGN2 (Figure 1d). Antibodies against HMGN1 and HMGN2 were raised against a 15-amino-acid peptide from the C-terminus of each protein and affinity purified (Eurogentec). They are specific to their respective HMGN proteins (results not shown). Antibodies 2752 and 2751 raised against HMGN3 internal peptides have been described previously [9]. Anti-H3K14ac (acetylated Lys¹⁴ of histone H3) (07–353), anti-H3K9ac (acetylated Lys⁹ of histone H3) (07–352) were from Upstate Biotechnology. Anti-H3K4me3 (trimethylated Lys⁴ of histone H3) was from Abcam (ab8580, Figure 3) or from Millipore (07–473, Figure 5), and normal IgG (I5006) was from Sigma.

Quantitative PCR

Real-time quantitative PCR was performed on the immuno-precipitated samples using the Stratagene Mx3000P machine and SYBR Green master mix (Roche). Primer pairs were designed across non-repetitive regions of the *Glyt1* gene, and checked for specificity and amplification efficiency. Primer sequences are listed in the Supplementary Online Data. PCRs contained either 0.19% of each immunoprecipitate or 0.5 ng of input DNA, and were carried out in duplicate. Enrichments were calculated as a ratio of the PCR readings in immunoprecipitate and input DNAs (ΔC_T). Log₂ ΔC_T values were converted into fold enrichments, taking into account the PCR efficiency of each primer pair. In Figures 2 and 3, fold enrichments for each experimental immunoprecipitation were normalized to the mean of the control IgG enrichments (mean calculated from all *Glyt1* primer pairs), to control for variation in immunoprecipitation efficiency between chromatin preparations. In Figures 5 and 6, fold enrichments were normalized to those for H3 at each primer set. For each histogram shown (i.e. for each antibody), the data are scaled so that the enrichment in control cells at primer set –1914 is set to 1.

Gel-retardation and *in vitro* acetylation assays

Gel-retardation assays were performed with nucleosome core particles and recombinant HMGN3 protein as described previously [9]. *In vitro* acetylation assays were performed in HAT buffer [50 mM Tris/HCl, pH 8.0, 10% glycerol (v/v), 1 mM DTT (dithiothreitol), 0.1 mM EDTA and 5 mM butyric acid]. Each 10 μ l reaction contained: 0.1 mg/ml nucleosome cores, 0.2 mM [14 C]acetyl-CoA (20 nCi), 100 ng of recombinant PCAF (Upstate Cell Signalling Solutions, 14–309), and various amounts of HMGN (added at specific molar ratio to core particles varied from 0.2 to 3.2). The assay was performed at 37 °C for 30 min. The reactions were stopped by the addition of an equal volume of an SDS-gel sample buffer [100 mM Tris/HCl (pH 6.8), 200 mM DTT, 2% SDS, 0.1% Bromophenol Blue and 20% glycerol], denatured for 5 min at 95 °C, and the proteins were resolved by SDS/PAGE (15% gel). The gels were stained with Coomassie Blue for estimation of protein quantities, soaked in enlignening enhancer solution (Dupont) for 30 min and vacuum dried. The radioactivity incorporated into the protein bands was visualized by a PhosphorImager (Molecular Dynamics) and quantified with ImageQuant software.

RESULTS

The relationship between HMGN3 binding and histone modifications across the *Glyt1* gene

In order to study the relationship between HMGN3 and histone modifications at the *Glyt1* gene, we generated Hepa-1 cell lines in which either HMGN3a, or its C-terminally truncated splice variant, HMGN3b (Figure 1a), is ectopically expressed. The new cell lines are named H-N3a and H-N3b respectively. Real-time RT-PCR and Western blotting showed that the control (parent) cell line had undetectable levels of HMGN3 (Figures 1b and 1d). Western blotting using the antibody 2752, which recognizes an internal peptide common to both HMGN3 splice forms (Figure 1a) [9], revealed similar levels of HMGN3b and HMGN3a proteins in the H-N3b and H-N3a cell lines respectively (Figure 1d). The N3-Cter antibody was raised against a peptide in the C-terminal RD of HMGN3a, and so did not detect HMGN3b protein (Figure 1d). Real-time RT-PCR indicated that the *Hmgn3b* and *Hmgn3a* transgenes are expressed in H-N3b and H-N3a cells respectively, at levels comparable with those of other *Hmgn* family members (Figures 1b and 1c). The levels of endogenous *Hmgn1* and *Hmgn2* mRNA were altered by less than 20% by the ectopic expression of *Hmgn3a* or *Hmgn3b* (Figure 1e).

We used ChIP assays to investigate the profile of HMGN3 binding across the *Glyt1* locus. The locus is approximately 35 kb long, and *Glyt1* expression can be driven from the *Glyt1a* or *Glyt1b* promoters, resulting in two splice forms with different 5' exons [36]. In Hepa-1 cells, the *Glyt1a* promoter is active, but the promoter for the alternative splice form, *Glyt1b*, is inactive (Figure 2a). ChIP assays using the N3-Cter antibody revealed HMGN3a binding across the entire *Glyt1* gene body in H-N3a cells (Figure 2a). The average relative enrichment for HMGN3a across the locus in H-N3a cells is 17-fold, compared with an average enrichment of 0.5-fold in the control cell line (Figure 2a), and 0.56-fold for non-immune IgG (results not shown). ChIPs with antibodies against internal HMGN3 peptides (2751 and 2752) generated a similar binding profile (Figure 2b), although the average enrichment across the locus was lower, at 4.7-fold. This may reflect the reduced accessibility of the internal epitopes compared with the C-terminal epitope [37].

The binding profile of HMGN3b in H-N3b cells was assayed using antibodies 2751 and 2752. The data show that HMGN3b is bound across the *Glyt1* gene body with an average enrichment of 5.5-fold, which is comparable with the enrichment of HMGN3a in H-N3a cells (Figure 2b). The precise pattern of peaks and troughs varied between the HMGN3a and HMGN3b profiles. Both profiles had small peaks at the TSS (transcription start site) and the

inactive *Glyt1b* start site, but peaks approximately 7 and 21 kb into the gene were more variable. Despite these differences, it is clear that ectopic HMGN3a/b expression in H-N3a/b cells leads to HMGN3a/b binding across the *Glyt1* gene body, and there is no major peak at the promoter or TSS.

In order to investigate whether histone modifications may be involved in recruiting HMGN3, or whether HMGN3 affects the levels of certain modifications, we profiled H3K14ac, H3K9ac and H3K4me3 across the *Glyt1* locus in the different cell lines. The level of H3K14ac across *Glyt1* was significantly increased in cells overexpressing HMGN3a or HMGN3b (Figure 3a). In control cells, small peaks of H3K14ac were found at the TSS and the 3' end of the gene, with an average enrichment of 1.6-fold across all points. In H-N3a and H-N3b cells, H3K14 acetylation was increased across the gene, resulting in average enrichments of 4.9- and 4.5-fold respectively. The peaks of H3K14 acetylation at the 5' and 3' ends of the gene were increased, and additional peaks within the gene body are also apparent. Interestingly, the shape of the H3K14ac binding profile in H-N3a cells is similar to that of HMGN3a (C-ter antibody), which is reflected in a moderate correlation coefficient of 0.66 between the two data sets. For comparison, the correlation between the IgG profile (results not shown) and H3K14ac in these cells is negligible, at -0.18. These data suggest that binding of HMGN3a or HMGN3b to chromatin *in vivo* leads to increased H3K14 acetylation either on the same nucleosome or adjacent nucleosomes.

H3K9ac and H3K4me3 were both highly enriched in tight peaks at the TSS, and H3K9ac was also present in a clear peak near the 3' end of the gene (Figures 3b and 3c). These profiles are distinct from that of HMGN3, suggesting that H3K9ac and H3K4me3 do not have a significant role in HMGN3 recruitment. Furthermore, the peaks do not change in cells overexpressing HMGN3a or HMGN3b, indicating that HMGN3 binding does not alter the levels of H3K9ac or H3K4me3.

HMGN protein binding at multiple genomic locations

The recent study by Cuddapah et al. [26] revealed HMGN1 peaks at the enhancers and/or promoters of many active genes. Enhancers have been defined as DHSs enriched in H3K4me1 (monomethylated Lys⁴ of histone H3) and p300 [38–40]. In order to identify any putative enhancers of the *Glyt1* gene, we used the UCSC genome browser [41] to examine the genomic context of the *Glyt1* gene and to compare it with publicly available ChIP-seq data from the ENCODE project [42,43]. As can be seen in Figure 4, there are several genes immediately downstream of *Glyt1*, whereas the upstream region is intergenic. Notably, at just over 5 kb upstream of the *Glyt1* TSS there is a putative enhancer region that is DNase I hypersensitive and enriched for H3K4me1, p300 and CTCF (CCCTC-binding factor) in most of the tissues studied.

We used ChIP to investigate whether HMGN3a is bound at the putative *Glyt1* enhancer and at other regions outwith the *Glyt1* gene. As shown in Figure 5(a), HMGN3a binding in H-N3a cells was comparable at all genomic locations studied: the upstream intergenic region, the putative enhancer, the *Glyt1* promoter, the *Glyt1* gene body, the downstream *B4galt2* gene and two unrelated genes *Hbb-b1* and *CC11*. This reveals that HMGN3a is not specifically targeted to the *Glyt1* gene, and is not enriched at its promoter or putative enhancer.

H3K14ac was enriched at the *Glyt1* TSS in control cells, but not at the putative enhancer. Expression of HMGN3a led to an increase in H3K14ac levels at all locations tested, showing that the effect of HMGN3 on H3K14 acetylation is not limited to the *Glyt1* gene body. Peaks of H3K9ac and H3K4me3 were observed at the putative enhancer (primer set – 5394) as well as at the TSS (primer set 312) (Figures 5b and 5c). The heights of these peaks

were the same in the control cells as in the H-N3a cells, providing additional evidence that HMGN3a does not alter the levels of these modifications.

HMGN1 and HMGN2 are the most predominant HMGN isoforms in the parent Hepa-1 cell line, and their mRNA levels are not altered by the ectopic expression of HMGN3 (Figure 1). ChIP was used to determine whether these isoforms are found in peaks at the *Glyt1* enhancer or promoter, and to investigate whether their binding profiles are altered by HMGN3 expression. Figures 6(a) and 6(b) show that HMGN1 and HMGN2 binding is comparable at all genomic locations tested, with no peaks at the enhancer, promoter or TSS of *Glyt1*. The enrichment of HMGN1 decreased by an average of 25% ($\pm 10\%$) in the H-N3a cells compared with the control cells. Conversely, HMGN2 enrichment increased by an average of 30% ($\pm 20\%$) in H-N3a cells. These data show that all three HMGN isoforms can bind at the *Glyt1* locus, but that none of them are specifically enriched at the regulatory elements.

HMGN3 and histone acetylation

To investigate how HMGN3 expression and histone acetylation correlates with *Glyt1a* transcription, we quantified the levels of *Glyt1a* mRNA in the different cell lines (Figure 7a). *Glyt1a* expression is increased by 2.3- and 2.6-fold in H-N3b and H-N3a cells respectively, suggesting that either the HMGN3 binding and/or the increased H3K14ac levels have contributed to increased transcription from the *Glyt1* locus. To investigate whether histone acetylation alone can affect *Glyt1a* expression, control cells were treated with the HDAC inhibitors sodium butyrate or trichostatin A for 3 h (Figure 7b). These treatments induced *Glyt1a* expression by 2.3- and 1.9-fold respectively, indicating that histone acetylation can stimulate *Glyt1a* expression in the absence of HMGN3.

Western blotting was performed to determine whether the increased H3K14 acetylation detected at the *Glyt1* locus in H-N3b and H-N3a cells (Figure 2) was also observed in bulk chromatin. The results show that global levels of H3K14ac are actually decreased in H-N3b and H-N3a cells relative to the control cells (Figures 8a and 8b). H3K9ac levels were not significantly altered, in agreement with the ChIP data. Furthermore, levels of the HATs (histone acetyltransferases) (*Gcn5*, *p300* and *Pcaf*) are unchanged or reduced in H-N3b and H-N3a cells compared with control cells (Figure 8c), showing that HMGN3 does not increase the expression of these chromatin modifiers. These results indicate that the increase in H3K14 acetylation over the *Glyt1* locus is unlikely to be due to indirect effects of HMGN3 on HAT or HAT co-factor expression.

One of the main HATs that acetylates H3K14 is PCAF, and previous reports have shown that other HMGN family members can stimulate histone H3 acetylation by PCAF *in vitro* [15,16]. To investigate whether HMGN3a and HMGN3b can also stimulate histone acetylation *in vitro*, HMGN3a and HMGN3b were first titrated against nucleosome core particles and the binding efficiency monitored by gel-retardation assays (Figure 9a). The results indicated that both HMGN3a and HMGN3b bind to nucleosomes with efficiency similar to HMGN1, as has been previously reported [9]. Pre-bound HMGN3–nucleosome complexes were then incubated with purified PCAF and radiolabelled acetyl-CoA. It is known that the main residue acetylated by PCAF is H3K14 [44]. After SDS/PAGE, ^{14}C incorporation into histones was quantified as a measure of acetylation activity (Figures 9b and 9c). These data show that HMGN3a can stimulate acetylation of nucleosomal H3 by up to 2-fold, whereas HMGN3b, which lacks the RD, does not have any effect in this assay. This result is in agreement with previous findings that the RD of HMGNs can enhance PCAF-mediated acetylation of H3K14 [16].

DISCUSSION

In the present study, we used the *Glyt1* locus as a model system to study the relationship between HMGN proteins, active histone modifications and gene expression. We confirmed that *Glyt1* is a target of HMGN3, as *Glyt1a* expression is increased when HMGN3 is ectopically expressed. However, HMGN3 is not enriched in specific peaks at the gene promoter, TSS or putative enhancer. Instead, it is bound throughout the gene locus, and is found at similar levels at other unrelated genomic locations. HMGN1 and HMGN2 were also bound at fairly constant levels at all the genomic regions tested. We found that ectopic expression of HMGN3a or HMGN3 leads to increased H3K14ac levels at all locations. In contrast, levels of H3K9ac and H3K4me3 enrichment at the *Glyt1a* promoter and putative enhancer are unaffected. *In vitro* experiments showed that HMGN3a, but not HMGN3b, is able to stimulate the ability of PCAF, one of the main H3K14 acetyltransferases, to acetylate nucleosomal H3. The results indicate that HMGN3a binding leads to a direct increase in H3K14 acetylation, and a concomitant increase in *Glyt1a* transcription. A role for histone acetylation in *Glyt1a* regulation is supported by the demonstration that treatment with HDAC inhibitors also leads to an increase in *Glyt1a* expression *in vivo*.

HMGN binding across the *Glyt1* locus

A recent study profiled genome-wide HMGN1 binding in human CD4⁺ T-cells [26], and found that HMGN1 is preferentially bound at the promoters of active genes. In addition, many of the HMGN1 peaks overlapped with DHSs and were enriched in active chromatin marks such as H3K4me3 and H2A.Z [26]. In contrast with these genome-wide data, we found that HMGN1, HMGN2 and HMGN3 are bound fairly evenly across the 35 kb *Glyt1* locus, the putative enhancer, upstream intergenic regions and at other unrelated genes.

We observed no correlation between HMGN binding and the H3K4me3 and H3K9ac modifications that mark the promoter and putative enhancer. These results indicate that HMGN proteins are not specifically recruited by the histone modifications usually found at active gene promoters and enhancers. This is consistent with previous studies on individual genes, which have observed a variety of HMGN-binding profiles. In the case of *Glut2* (glucose transporter 2), HMGN3 is enriched in a 3 kb region at the promoter, and this enrichment requires the transcription factor Pdx1 [23]. Knockdown of HMGN3 reduces the binding of several key transcription factors to the *Glut2* promoter, reduces H3K14 acetylation levels, and reduces *Glut2* expression by approximately 80% [23]. Peaks of HMGN1 binding are also found at three regions of *Sox9*, although not including the promoter, and HMGN1 is important for repressing *Sox9* expression in mouse limb bud cells [24]. In contrast, an even distribution of HMGN1 binding was observed across the *Hsp70* locus, and HMGN1 was shown to be important for the heat shock-induced nucleosome remodelling and acetylation at the *Hsp70* promoter [25]. Our data indicate that the transcription factors and/or chromatin structural elements required for enhanced HMGN recruitment are absent from the *Glyt1* locus in Hepa-1-derived cells. It will be interesting to investigate whether the HMGN peaks observed at regulatory elements on a genome-wide scale [26] are due to recruitment by specific transcription factors, or by some other characteristic of open/actively transcribed chromatin.

There does appear to be a relationship between the peaks of HMGN3a/b and H3K14ac and the *Glyt1* exons, although there are not enough primer sets to draw any firm conclusions. This relationship would be consistent with the genome-wide analysis [26], which revealed that HMGN1 is enriched at the intron–exon boundaries of expressed genes. It is well established that nucleosomes are well positioned relative to intron–exon boundaries, and that nucleosomes within exons are enriched in active histone modifications [45]. It is thought

that chromatin structure might influence the rate of RNA polymerase II movement and exon usage during co-transcriptional splicing [45].

Stimulation of H3K14 acetylation by HMGN3a and HMGN3b

The ability of HMGN3a to stimulate H3K14 acetylation *in vitro* is consistent with previous data showing that HMGN2 and HMGN1 can stimulate the ability of PCAF to acetylate nucleosomal H3 by 3.5-fold and 2-fold respectively [15,16]. Kinetic data indicated that HMGN1 promotes H3K14 acetylation by making the H3 tail a better enzymatic substrate, rather than by increasing the binding affinity of PCAF or by decreasing the action of a specific deacetylase [15]. The RD is required for both HMGN1 and HMGN2 to stimulate acetylation by PCAF [15,16]. The RD is absent from HMGN3b, which explains why we could not detect HMGN3b-mediated stimulation of acetylation of mononucleosomes *in vitro*. It is notable that while H3K14 acetylation is increased by HMGN3a, acetylation of the nearby residue, H3K9, is unaffected *in vivo*. Similar results were observed for HMGN1, which stimulates H3K14 acetylation, yet inhibits H3S10 phosphorylation and H3K9 acetylation [15–17,22].

Given that HMGN3b does not stimulate nucleosomal acetylation by PCAF *in vitro*, it is unclear as to why it should promote H3K14 acetylation across the *Glyt1* locus *in vivo*. It is possible that HMGN3b can alter the structure of the H3 tail in chromatin to promote acetylation by PCAF, whereas in mononucleosomes this is not possible. Indeed, it has previously been shown that the acetylation activity of recombinant PCAF increases in proportion to the length of the nucleosomal array substrate [46]. It is also possible that the recombinant PCAF used these *in vitro* experiments has a different response to HMGN3b compared with the various PCAF, GCN5 and Elp3 complexes that can acetylate H3K14ac *in vivo* (see below). Alternatively, HMGN3a and/or HMGN3b may alter the chromatin structure of the *Glyt1* locus by another mechanism, for example by competing with the linker histone H1 [11,47], which could increase accessibility to HATs and thus promote transcription [11,47].

Acetylation of H3K14 across the *Glyt1* gene body increases transcription

The relationship between histone acetylation and transcription is well documented. H3K14 acetylation is regulated by a balance of acetylation and deacetylation activities [48]. In yeast, H3K14 acetylation on gene bodies is deposited by GCN5 within the SAGA complex [49–51], and by Elp3 within the elongator complex [52], whereas Clr3 is the main H3K14 deacetylase [51]. In metazoans, GCN5 and its close homologue, PCAF, are found in several highly related 2 MDa complexes (the TFTC, STAGA and PCAF/GCN5 complexes) as well as the 700 kDa ATAC complex (reviewed in [53]). These complexes have been shown to acetylate nucleosomal H3K14, in addition to other residues, although it is clear that subunit composition influences the substrate specificity of the HAT complex [53,54].

On highly transcribed genes, H3 acetylation by SAGA is linked to nucleosome eviction and thus increased elongation efficiency [50]. However, it has also been shown that genes transcribed at lower levels do not lose nucleosomes during transcription, and that the level of acetylation across these genes is closely correlated with the rate of transcription [50]. This is consistent with the results of the present study, where the level of H3 acetylation is related to the level of *Glyt1* transcription. The nucleosome remodelling complex RSC is known to be recruited by H3K14 acetylation via its bromodomains [55], and has been shown to increase elongation efficiency by RNA polymerase II *in vitro* [56]. Furthermore, prior acetylation of chromatin by SAGA increases the ability of RSC to stimulate transcription elongation [56,57].

We suggest that H3K14 acetylation across the *Glyt1* gene body may improve elongation efficiency, possibly by promoting the recruitment of the nucleosome remodelling complex RSC. HMGN1 has previously been shown to stimulate RNA polymerase II elongation on isolated SV40 minichromosomes [58], but a link between HMGNs and transcription elongation *in vivo* has not been demonstrated. The *Glyt1a* primary transcript is relatively long (35 kb), and straddles the silent *Glyt1b* promoter, so it may be particularly sensitive to improvements in elongation efficiency.

In summary, our analysis has revealed that although HMGN3 expression stimulates *Glyt1* transcription, its binding is not specifically enriched at the gene body or its regulatory elements. HMGN3 expression does not alter the level of the histone modifications associated with the TSS, but does increase the level of H3K14ac across the locus. We propose a model whereby HMGN3-stimulated H3K14 acetylation across the bodies of genes such as *Glyt1* can lead to more efficient transcription elongation and increased mRNA production.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

ChIP	chromatin immunoprecipitation
CTCF	CCCTC-binding factor
DHS	DNase I-hypersensitive site
DTT	dithiothreitol
ERα	oestrogen receptor α
Gapdh	glyceraldehyde-3-phosphate dehydrogenase
GCN5	general control of amino acid synthesis 5
Glut2	glucose transporter 2
Glyt1	glycine transporter 1
H3K4me1	monomethylated Lys ⁴ of histone H3
H3K4me3	trimethylated Lys ⁴ of histone H3
H3K9ac	acetylated Lys ⁹ of histone H3
H3K14	Lys ¹⁴ of histone H3
H3K14ac	acetylated Lys ¹⁴ of histone H3
H3S10	Ser ¹⁰ of histone H3

HAT	histone acetyltransferase
HDAC	histone deacetylase
HMGN	high-mobility group nucleosome-binding
Hsp70	heat-shock protein 70
MSK1	mitogen- and stress-activated kinase 1
NBD	nucleosome-binding domain
NLS	nuclear localization sequence/signal
PCAF	p300/CREB (cAMP-response-element-binding protein)-binding protein-associated factor
PDX-1	pancreatic and duodenal homeobox-1
PITX2	pituitary homeobox 2
RD	regulatory domain
RT	reverse transcription
RXR	retinoid X receptor
SRF	serum-response factor
TSS	transcription start site

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Figure 1. Overexpression of *Hmgn3b* and *Hmgn3a* in Hepa-1 cells

(a) Schematic diagram of HMGN3a and its shorter splice form, HMGN3b. The two NLS, the NBD and the RD are indicated. The locations of peptides used to raise antibodies 2751, 2752 and N3-Cter are indicated. (b) *Hmgn3a* and *Hmgn3b* were overexpressed in H-N3b and H-N3a cells respectively, and were barely detectable in control cells. *Hmgn3* expression was quantified by real-time quantitative PCR and is plotted as a percentage of *Gapdh* expression. (c) *Hmgn3a* mRNA was expressed at levels comparable with those of *Hmgn1* and *Hmgn2* in H-N3a cells. (d) HMGN3b and HMGN3a proteins were produced at similar levels in H-N3b and H-N3a cells respectively. Acid-soluble proteins from the three cell lines were run on three parallel SDS/PAGE gels. Left-hand panel: a strip from the Coomassie Blue-stained protein gel showing the relative protein levels in the extracts. The bands shown are the most prominent on the gel, and correspond to the linker histone variants. The positions of the 26 and 34 kDa size markers are indicated. Middle and right-hand panels: Western blots were probed with antibodies 2752 and N3-Cter. No other bands were visible on the blots. Quantification of the bands in the 2752 blot revealed relative intensities of 1.73:1 for HMGN3b/HMGN3a. (e) *Hmgn1* (left-hand histogram) and *Hmgn2* (right-hand histogram) mRNA levels are unchanged in H-N3b and H-N3a cell lines compared with the control. *Hmgn* mRNA was normalized to *Gapdh* and is plotted relative to expression in the control cells (con).

Figure 2. HMGN3a and HMGN3b are bound across the *Glyt1* locus

(a) ChIP profiles comparing HMGN3a binding across the *Glyt1* locus in H-N3a cells and control cells. ChIPs were carried out using the N3-Cter antibody (means±S.E.M., $n=2$ ChIP replicates). Numbering starts at the first transcribed base pair. The relative positions of the TSS and 14 exons for *Glyt1a* (active) are shown in the schematic diagram above the graph. The first exon for *Glyt1b*, which is not expressed in Hepa cells and is not included in the *Glyt1a* transcript, is also shown. (b) ChIP profiles using internal HMGN3 antibodies (2751 and 2752 combined) in H-N3a, H-N3b and control cells. Points in the control cell profile represent the mean and S.E.M. from three ChIP replicates.

Figure 3. H3K14 acetylation across the *Glyt1* locus is increased in cells expressing *Hmgn3a* or *Hmgn3b*

ChIP profiles for (a) H3K14ac, (b) H3K9ac and (c) H3K4me3 across the *Glyt1* locus in control, H-N3b and H-N3a cells (means $\pm$ S.E.M., $n=2$ ChIP replicates).

Figure 4. ENCODE data for the *Glyt1* locus and surrounding regions

Screenshot from the UCSC genome browser [41] of data generated by the Ren laboratory (Ludwig Institute for Cancer Research, USCD) as part of the ENCODE consortium (genome build NCBI37/mm9) (<http://genome.ucsc.edu/>) [42,43]. The locations of *Glyt1* and the two downstream genes, *Ccdc24* and *B4galt2* are indicated. ChIP-seq data from mouse cerebellum for DNase I hypersensitivity and for H3K4me1, H3K4me3 and CTCF are shown below, as is data for p300 in mouse heart tissue. The locations of the additional primer sets used in Figure 5 are indicated at the bottom. Primer set -5394 is located at a putative enhancer, as indicated by the peaks of H3K4me1 and p300 and the DHS [38–40].

Figure 5. HMGN3a binding is not limited to the *Glyt1* gene body

Relative enrichment for (a) HMGN3a (b) H3K14ac (c) H3K9ac and (d) H3K4me3 at various sites outwith the *Glyt1* gene body in control cells and H-N3a cells. Locations of numbered primer sets near the *Glyt1* locus are shown in Figure 4. *B4galt2* is a gene downstream of *Glyt1* as shown in Figure 4. *Hbb-b1* is the β -globin adult major gene and *Ccl1* is a chemokine gene, both of which are repressed in Hepa-1 cells. HMGN3 represses the tumour necrosis factor α (TNF α)-induced expression of *Ccl1* in mouse embryonic fibroblasts (K.L. West, unpublished work). ChIP enrichments were normalized to H3 enrichment for each primer set.

Figure 6. HMGN1 and HMGN2 are enriched to similar levels at all genomic locations tested
Relative enrichment for HMGN1 (**a**) and HMGN2 (**b**) in control cells and H-N3a cells at the same genomic locations tested in Figure 5. Enrichments were normalized to H3 for each primer set.

Figure 7. *Glyt1a* expression is increased in cells expressing *Hmgn3a* or *Hmgn3b*, and in cells treated with HDAC inhibitors

(a) Expression of *Glyt1a* mRNA in control, H-N3b and H-N3a cells. Expression was normalized to *Gapdh* and is plotted relative to that in control cells (means $\pm$ S.E.M., $n=2-4$ samples). Data using primers specific to the *Glyt1a* isoform is presented. Primers specific to exon 8 (further downstream) gave similar results (results not shown). *Glyt1b* expression was barely detectable above background and is not shown. (b) Expression of *Glyt1a* mRNA in control cells treated with either sodium butyrate (2 mM or 5 mM in water) or trichostatin A (165 nM in ethanol) for 3 h. Expression was normalized to *Gapdh* and is plotted relative to expression in cells treated with vehicle alone (water or ethanol).

Figure 8. Global acetylation levels are not altered in cells expressing *Hmgn3a* or *Hmgn3b*
(a) Western blots of chromatin from control, H-N3b and H-N3a cells, probed with antibodies against H3K14ac, H3K9ac or histone H3. **(b)** Band intensities from the blots in **(a)** were normalized to those of H3, and modification levels are plotted relative to those in control cells. **(c)** Expression of the HATs *Gcn5*, *p300* and *Pcaf* in the different cell lines was quantified by real-time quantitative PCR. Expression was normalized to *Gapdh* and is plotted relative to that in control cells.

Figure 9. HMGN3a stimulates the ability of PCAF to acetylate nucleosomal histone H3 *in vitro*
(a) Gel-retardation assays showing the extent of HMGN3 binding to nucleosome core particles. Nucleosome core particles were incubated with increasing amounts of purified recombinant HMGN1 (lanes 2–4), HMGN3a (lanes 5–7) or HMGN3b (lanes 8–10) in 2×TBE prior to electrophoresis on a 5% polyacrylamide gel. The molar ratios of HMGN to nucleosome core particle were 0 (lane 1), 0.8 (lanes 2, 5 and 8), 1.6 (lanes 3, 6 and 9) and 3.2 (lanes 4, 7 and 10). The expected stoichiometry of HMGN/nucleosome binding is 2:1.
(b) *In vitro* acetylation of nucleosomal H3 by PCAF. Nucleosome core particles were incubated with various amounts of HMGN protein, radiolabelled acetyl-CoA and PCAF. After electrophoresis, the gels were stained with Coomassie Blue for the estimation of protein quantities (upper panels) and exposed to a phosphorimaging plate for quantification of ¹⁴C incorporation. Arrowheads indicate the positions of HMGN3a and HMGN3b. HMGN3b co-migrated with H2A/H2B. **(c)** Quantification of the H3 acetylation assayed in **(b)**.