Differential effect of H1 variant overproduction on gene expression is due to differences in the central globular domain

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ABSTRACT

The in vivo overproduction of two mouse histone H1 variants in homologous mouse fibroblasts has opposite effects on gene expression. Overproduction of H1\(^0\) results in repression of transcript levels of all polymerase II genes tested. In contrast, overproduction of H1c results in elevated levels of transcripts. We created a series of chimeric H1 genes in which the regions encoding the three structural domains common to this family of these proteins were systematically switched. Overexpression of these genes in vivo resulted in the accumulation of large amounts of the chimeric H1 in chromatin. Analysis of the effects of overproduction of these proteins revealed that the differential effect of H1 variant overproduction on gene expression is due to differences in the central globular domain.

INTRODUCTION

The nucleosome is the fundamental repeating unit of eukaryotic chromatin (1–5). Two molecules each of the four core histones, H2a, H2b, H3 and H4, form an octamer around which is wrapped 146 bp of DNA. In higher eukaryotes, one molecule of a fifth histone, the linker or H1 histone is also present. Most H1 proteins have a tripartite structure consisting of a central, trypsin-resistant globular domain

or facilitate the condensation of nucleosomal DNA into 30 nm chromatin fibers (11,12).

The role of nucleosomes as general repressors of transcriptional initiation in vitro is well established (13,14). Genetic studies in yeast have provided convincing evidence that the core histones play an important role in gene regulation in vivo (15). Manipulation of histone stoichiometry or replacement of normal histone genes with mutated copies resulted in complex changes in the expression of a wide range of genes and lead to the identification of key structural features of these proteins. It was suggested some time ago that H1 may also function as a non-specific repressor of transcription (16). The involvement of H1 in transcriptional repression of Xenopus polymerase III genes has been clearly demonstrated (17–19). Studies involving H1–DNA complexes and chromatin reconstituted in vitro with nucleosome cores and H1 identified H1 as a transcriptional repressor and demonstrated that some transcription factors act as anti-repressors to antagonize H1-mediated repression (20–22). However, in vivo studies in Xenopus (23) and Tetrahymena (24) suggest that H1 is more specific in its action and limited to a subset of genes. A conservative view is that H1 can modulate the expression of some, but perhaps not all (25), genes by modifying the accessibility of promoter or enhancer sequences to transcriptional regulators. This might be accomplished by direct steric occlusion of binding (26), by limiting nucleosome mobility (27,28), by reducing transient dynamic exposure of DNA from the nucleosome surface (29,30), or by promoting condensation of chromatin into higher-order structures (31).

In higher organisms, the linker histones consist of multiple non-allelic variants and several lines of evidence suggest that a functional significance underlies this heterogeneity (32–34). During development in Xenopus the sequential expression of cleavage stage (B4), adult (H1) and differentiation-specific (H1\(^0\)) linker variants correlates with changes in chromatin structure as well as replicative and transcriptional capacity (35). In avian erythrocytes the accumulation of the tissue-specific H5 variant is associated with the establishment of a stable, inert chromatin structure (36,37). At least seven different H1 variants have been detected in mouse (38–40). Evidence that there is a functional significance to this diversity includes differences among the variants in their turnover rates (39), timing of synthesis during development (40), extent and schedule of phosphorylation with respect to the cell cycle (41) and in the ability to condense DNA and dinucleosomes (32,42,43). Whereas it is compelling to assume that there are functionally distinct classes of mammalian H1 variants, this has been difficult to demonstrate in vivo. Unfortunately the type of genetic replacement analysis that has been exploited in yeast is much more difficult in higher organisms due to the multiplicity of H1 genes and the presence of

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compensatory responses. As an alternative approach we developed a system for the overexpression of individual H1 variants and mutagenized constructs in homologous mouse 3T3 cells (44,45). We demonstrated significant differences with respect to cell cycle progression and gene expression following the overexpression of two variants, H1° and H1c (45). Transformants overproducing H1° exhibited transient inhibition of both G1 and S-phase progression and significantly reduced expression of all genes tested. This is consistent with the proposed role of this variant in the stabilization of inert chromatin in differentiated cells (46). In contrast, overproduction of H1c to comparable levels had no effect on cell cycle progression and actually resulted in increased transcript levels from some genes. These results support the suggestion that functional differences exist among H1 variants. Towards identifying the structural features responsible for these differences we created and overexpressed in mouse cells a set of chimeric H1 proteins in which the three structural domains of H1° and H1c were systematically switched. The results clearly identify the central globular domain as the most important determinant of the differential effect of these variants on gene expression.

MATERIALS AND METHODS

Construction of domain shift hybrids

Domain shift hybrids of the H1° and H1c genes are described in Figure 1B. They were constructed in multiple steps using polymerase chain reaction (PCR)-based and silent mutagenesis (insertion of restriction sites without altering coding capacity) using standard procedures (47). Junctions between the globular domain of either variant and the C-terminal domain of the other variant were created using NarI sites at codon 92 of H1° and codon 104 of H1c. For junctions between the N-terminus of H1c and the globular domain of H1°, a BglII site was introduced at codons 32 and 33 of H1c and a BamHI site was introduced at codons 20 and 21 of H1°. Ligation of the compatible ends of these sites generated the junction. For junctions between the N-terminus of H1° and the globular domain of H1c, a Nhel site was introduced at codons 22 and 23. Ligation of the Nhel sites created the junction. All constructs were verified by DNA sequencing. The coding region and short 5′ and 3′ flanking regions for each hybrid were placed in an expression vector containing the following: a 1.8 kb EcoR1–BglII fragment from pdBPVMMTneo (48) containing the mouse metallothionein (MT) I promoter (49), a 563 bp fragment (nt 1141–1703 relative to the transcriptional start site), including a cleavage/polyadenylation site, from the 3′ flanking region of the mouse MH143 gene (50,51), the large EcoR1–BamHI fragment of pSV2neo (52).

Cell culture

Mouse BALB/c 3T3 cells (clone A31 from the American Type Culture Collection) and all derived transformants were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum (Gibco BRL) at 37°C under 5% CO2. Hybrid constructs were transfected into 3T3 cells by the Ca3PO4 co-precipitation method.
Isolation of histones and separation of H1 variants by high performance liquid chromatography (HPLC)

Total histones were extracted as previously described (45) except that all crude chromatin pellets were resuspended in double the volume of 0.2 M H2SO4. Total histones were separated with a BioRad Model 2800 HRLC system with a 0.46 × 25 cm, 300 Å, 5 mM C-18 reverse phase column (catalogue no. 218BTP54, Vydac) as previously described (45). Protein elution was detected with a BioRad Bio-Dimension detector at 210 nm and peaks were quantitated using the BioRad HPLC Gradient software. As absorbance at this wavelength is most reflective of the number of peptide bonds, a correction was made for the size of each variant.

**Gel electrophoresis of H1 variants and hybrids**

All gel electrophoresis procedures used a BioRad mini-protein II slab cell system. SDS–polyacrylamide gels were run according to the procedure of Laemmli (54) at 12% acrylamide and methylene bisacrylamide (37.5:1). Acid polyacrylamide gels (pH 4.5) were run according to the procedure of Reisfeld et al. (55) as modified by Kistler and Geroch (56). Gels were fixed and stained with Coomassie Blue.

**RNase protection assays (RPA)**

Total cytoplasmic RNA was isolated as described (57). Each preparation was resuspended in the appropriate amount of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.8) to yield equal aliquots on a per cell basis. DNA probes for cyclophilin (58), β-actin (59) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (60) were obtained from Ambion, Inc. Probe RNA was synthesized and labeled with [α-32P]UTP using a Maxi-Script kit (Ambion) according to the manufacturer’s instructions. Full-length probes were purified and eluted from denaturing 5% polyacrylamide gels. RNase protection assays were carried out with an Ambion RPA-II kit as described by the manufacturer. Protected fragments were separated on denaturing 5% polyacrylamide gels, which were dried and exposed to X-ray film. Quantitation was with a Molecular Dynamics phosphorimager system. Control experiments in which the amount of added RNA was varied confirmed that probe was in excess and that the response was linear over the range tested.

**RESULTS**

**Isolation of transformants overproducing domain-shift hybrid H1s**

A comparison of the amino acid sequences of the H10 and H1c variants (Fig. 1A) indicates that sequence identity and similarity are fairly evenly distributed throughout these proteins. The N-terminal domain of H10 is considerably shorter than that of H1c (20 versus 32 aa). The basic C-terminal tail of H10 is 6 aa shorter than that of H1c. These proteins both have a predicted pl of 10.83. Although the globular domains of H10 and H1c display considerable sequence divergence they are expected to have similar three dimensional structures (see Discussion).

We created a series of mutant DNA constructs in which the domains of these variants were systematically switched (Fig. 1B). By analogy with the more well characterized avian variants (3) we considered the globular domain of H10 to consist of aa 21–94 and that of H1c to consist of aa 33–106. The switchpoint between the N-terminal domains and the globular domains was engineered to be precisely at the start of the globular domain. Due to a fortuitous common restriction enzyme site the switchpoint used between the globular and C-terminal domains was the common Phe residue (aa 92 of H10 and aa 104 of H1c). Thus the fragments we refer to as the C-terminal domains actually contain 2 aa from the globular domain of the respective variant. The variants differ conservatively at one of these positions (Arg93 in H10 and Lys105 in H1c).

We initially created all six possible domain switch combinations. However, one of these, C0C was subsequently found by DNA sequencing to contain an internal deletion and was not analyzed further. The remaining constructs were placed into an expression vector such that their transcription is under control of the heavy metal-inducible mouse MTI promoter (49). In this vector 3′-end formation is determined by a cleavage/polyadenylation signal derived from a distal 3′-flanking region of the mouse H1c gene (50,51) and results in a message that is stable in the presence and absence of DNA synthesis (45). The constructs were introduced into 3T3 cells and stable single colony transformants were isolated. Cultures of these transformants, as well as control 3T3, MTH10 and MTH1c lines, were induced to overproduce the introduced construct (which we refer to as exogenous) by addition of ZnCl2 during serum-starvation for four days. Total histones were extracted from crude chromatin preparations and separated by HPLC (Fig. 2). Transformants into which hybrid constructs were introduced displayed a marked increase in the HPLC peak corresponding to the endogenous variant which bears the same globular domain.

![Figure 2](image-url)
Figure 3. Separation and identification of domain switch hybrid H1 variants by SDS and acid gels. From each transformant the major peak of material from HPLC profiles similar to those shown in Figure 2 was collected, concentrated and run on SDS–polyacrylamide gels (A) or acid polyacrylamide gels (B).

This was expected as the HPLC procedure used separates variants on the basis of hydrophobicity, which is contributed primarily by the globular domain. To verify that the protein eluting in these peaks was the expected variant and to get an estimate of the relative amount of exogenous and endogenous variant in that peak, individual peaks were purified and run on SDS and acid polyacrylamide gels (Fig. 3). The migration pattern on SDS gels confirmed three of the hybrids, 0CC, 0C0 and C00. The major peak from MT0CC and MT0C0 eluted from HPLC at the H1c position (Fig. 2). The isolated material from these peaks migrated on SDS gels faster than H1c and more closely to H10 (Fig. 3A). This is consistent with the calculated MW of these hybrids (Fig. 1B). The major peak from MT0CC elutes from HPLC at the H1c position (Fig. 2). On SDS gels the purified material migrates more closely to that of H1c as expected. These results also show that at least 90% of the material in the major peak is the exogenous hybrid. The two other hybrids, CC0 and 00C, migrate on SDS gels in the same position as the endogenous variant from the HPLC fraction, as expected. To confirm these hybrids we used acid gels. The major peak from MTCC0 eluted at the H1c position (Fig. 2) but migrated on acid gels at a position distinct from either H10 or H1c (Fig. 3B). The major HPLC peak from MT00C eluted at the H10 position but migrated slightly more slowly than H10 on acid gels. Interestingly this hybrid displays a minor breakdown or oxidation product similar to what we routinely observe with H10 (44). The minor product from MT00C runs distinct from that of H10. The migration patterns of material from MT0CC and MT0C0 on acid gels were distinct from that of H10 and H1c confirming the results from SDS gels. We conclude from this analysis that each of the transformants is producing a large amount of the expected exogenous H1 hybrid. The material collected for HPLC analysis was from crude chromatin preparations indicating that these hybrids are assembled into chromatin during the induction period.

**Effect of overproduction of hybrid H1s on gene expression**

We tested the effect of overproduction of these hybrids on the expression of a series of genes that give the signature response to overproduction of the wild-type variants, that is repression by H10 and activation by H1c (45). For this experiment cultures were starved and induced for only 3 days. While this protocol results in slightly lower levels of overproduction of the exogenous variants, the cells appear healthier, and >95% are able to re-enter the cell cycle upon release (data not shown). At the end of the induction period total histones and total cytoplasmic RNA were isolated from parallel cultures. H1 variants were separated by HPLC (Table 1). The steady state levels of transcripts from the cyclophilin, β-actin and GAPDH genes were determined by RPA analysis (Fig. 4). The results were straightforward. Transforms overproducing H1 variants containing the globular domain of H1c (MTH1c, MT0CC, MT00C and MTC00) displayed levels of these transcripts 2–3-fold higher than those of control 3T3 cells. Transformants overproducing H1 variants containing the globular domain of H10 (MTH10, MT00C and MTC00) displayed either a slight to moderate repression of these genes or showed no difference from control cells. rRNA levels were not greatly affected in any of the transformants.

<table>
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<th>Cell line</th>
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<th>H10</th>
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<th>H1d/e</th>
<th>H1c</th>
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*Data were derived from HPLC profiles of histones isolated from the experiment described in Figure 4. Data for each variant are presented as the percentage of the total area under all peaks, corrected for size as described in Materials and Methods.

*bFor cell lines expressing hybrid H1 variants the endogenous variant that elutes with the hybrid cannot be quantitated. These variants are assumed to make up a small portion of the peak assigned to the hybrid H1.
variants were obtained (80–90%; see Fig. 2). RPA analysis of increased and higher levels of overproduction of exogenous negligible. In other experiments, the induction/starvation time was probably repressive in the sense that chromatin containing any H1 of transcription, we embrace the view that linker histones are although H1s are considered by many to act as general repressors in vivo our original observation that the H1 variants may differ in the degree to which is less transcriptionally competent than H1-deficient chromatin probably repressive in the sense that chromatin containing any H1 that individual H1 variants may differ in the degree to which they are repressive. Our original observation that the in vivo overproduction of two variants, H10 and H1c, had different effects on cell cycle progression and gene expression supports this view (45). The results presented here indicate that this difference lies in structural differences in the central globular domain. This result was somewhat unexpected as the most obvious difference between these variants is the shorter N- and C-terminal tails of H10 relative to H1c. Differences in the effects of H1 variants on chromatin structure during embryogenesis and spermatogenesis have generally been attributed to differences in the length and charge density in the tail domains (35). The central globular regions of H10 and H1c are expected to be very similar (61,62). This implies that subtle differences in the structures of this region and perhaps the manner in which they interact with the nucleosome have important consequences on chromatin function.

Recently the crystal structure of the globular region of the extreme variant H5 (GH5), which is closely related to H10, has been solved (61). The structure identifies this protein as a member of the family of DNA-binding proteins exhibiting a modified helix-turn-helix motif known as the ‘winged helix’ (63,64). Strong structural similarities were noted between GH5 and catabolite activator protein (61,65) and the liver-specific transcription factor HNF-3γ (66), both of which have been crystallized in a complex with DNA. The predicted structure of the main chicken H1 protein (derived primarily from NMR data) has also been fitted to this structure (62). A sequence comparison of the globular regions of chicken H5 (GH5), chicken H1 (GH1) and those of our H10 and H1c variants together with the predicted sites of secondary structure is shown in Figure 6. Considering the very high degree of sequence identity between H10 and H5, and between H1c and GH1 in these regions, it is reasonable to assume similar structural features exist in the murine variants.

Based on the structures and their similarity to sequence-specific DNA binding proteins as well as biochemical data (67–69) a primary DNA binding site consisting of helix III and the adjacent β-hairpin has been proposed. Helix III corresponds to the recognition helix of the sequence-specific factors CAP and HNF-3γ. Although GH5 has not been crystallized in a complex with DNA, its structure suggests that a similar mode of binding occurs in the nucleosome. Included in this site are the basic residues Lys69, Arg73 and Lys85 (numbering according to GH5) which are conserved in all four variants. However, a considerable amount of sequence divergence has occurred in Helix III. These may lead to important consequences for the mode or strength of DNA binding and possibly for sequence preference. While H1s do not have strict sequence specificity, they do show sequence preferences and evidence that this preference may differ among DISCUSSION

Although H1s are considered by many to act as general repressors of transcription, we embrace the view that linker histones are probably repressive in the sense that chromatin containing any H1 is less transcriptionally competent than H1-deficient chromatin but that individual H1 variants may differ in the degree to which they are repressive. Our original observation that the in vivo overproduction of two variants, H10 and H1c, had different effects on cell cycle progression and gene expression supports this view (45). The results presented here indicate that this difference lies in structural differences in the central globular domain. This result was somewhat unexpected as the most obvious difference between these variants is the shorter N- and C-terminal tails of H10 relative to H1c. Differences in the effects of H1 variants on chromatin structure during embryogenesis and spermatogenesis have generally been attributed to differences in the length and charge density in the tail domains (35). The central globular regions of H10 and H1c are expected to be very similar (61,62). This implies that subtle differences in the structures of this region and perhaps the manner in which they interact with the nucleosome have important consequences on chromatin function.

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Figure 4. Effect of overproduction of H1 variants and domain switch hybrids on gene expression. Cultures were incubated for 3 days, with daily changes, in medium containing 0.5% calf serum and 50 µM ZnCl2, prior to the isolation of total cytoplasmic RNA. (A–C) Transcript levels were determined by RPA analysis as described in Materials and Methods. Total RNA from equal numbers of cells from each culture was assayed. The lanes labeled P contain a diluted (1:20) aliquot of labeled probe alone. The lanes labeled C contain controls of labeled probe, without addition of cellular RNA, treated with RNase. (D) RNA was electrophoresed on a 0.9% agarose gel and stained with ethidium bromide.

The results from multiple RPA determinations from a single experiment are quantitated in Figure 5 and are representative of those routinely obtained with a 3 day induction protocol. It should be noted that the increased levels of expression of the cyclophilin, actin and GAPDH genes occurs at only moderate levels of overproduction of the exogenous variant (48–65%; Table 1). With this protocol the repressive effect of overproduction of variants bearing the globular domain of H10 is moderate or negligible. In other experiments, the induction/starvation time was increased and higher levels of overproduction of exogenous variants were obtained (80–90%; see Fig. 2). RPA analysis of transcript levels under these conditions indicated that the repressive effect of exogenous variants bearing the globular domain of H10 was more severe. In addition, these cultures displayed a transient delay in cell cycle entry, as predicted (45, data not shown).

DISCUSSION

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Figure 5. Quantitation of transcript levels relative to those of control 3T3 cells. Data are from Figure 4 and additional RPA analyses. Error bars represent the standard deviation from at least three determinations.
different variants has been obtained (70,71). Also included in this proposed primary site are His25 and His62 which are found just upstream of Helices I and III, respectively. These residues in GH5 can be crosslinked to DNA in chromatin (67). Interestingly, a His residue is found at the corresponding position of His25 in H10 but is replaced in chicken H1 and mouse H1c by a Gly residue.

A proposed secondary binding site (61,69) including the conserved residues Lys40, Arg42, Lys52 and Arg94 is located on the opposite side of GH5. In H5 and H10 a neutral Asn residue is conserved residues Lys40, Arg42, Lys52 and Arg94 is located on the opposite side of GH5. In H5 and H10 a neutral Asn residue is located between Lys40 and Arg42 while in H1c and chicken H1 a Glu residue is found. This acidic amino acid might lessen the charge density in this region and reduce DNA binding affinity of H1c. Subtle but important differences between GH1 and GH5 were noted in the loop between the second and third helices suggesting that this region might be responsible for the difference in affinity for DNA between these two variants (62). Notably Lys59 of GH5 is conserved in H10 but is replaced in GH1 and H1c by an acidic Asp residue. Finally, Tyr53 and His57, which appear to be located at the interface of the crystallized GH5 dimers are conserved in H10 but replaced by Ala or Gly residues in GH1 and H1c. It was suggested that this might explain why H5 but not H1 forms dimers in solution (62). Whether this has consequences for H1 interactions in chromatin is unclear.

Any or all of these differences could contribute to the differential behavior of these variants in vivo. There are several possible ways in which binding of the globular domain of H1 to nucleosomes might affect gene expression. Considering the structural similarities between transcription factors and the globular domain of H1 a direct competition for occupancy of sites might occur. H1 has also been shown to limit nucleosome mobility and transient dynamic exposure of DNA from the nucleosome surface. If the globular domain of H1 interacts with two strands of DNA (69) at or near the dyad axis of the nucleosome it is easy to envision how this would restrict mobility or transient dynamic exposure.

Numerous studies indicate that H1 is under-represented in active chromatin or that the manner in which H1 binds to this region is altered (see 33 for review). Protein–DNA cross-linking studies showed that in active genes the globular domain of H1 could not be cross-linked to DNA but the tails remained attached (72). Disruption or modification of H1 binding to nucleosomes via the central globular domain may be a necessary step in activation of many genes; how this is accomplished is unclear. Whether molecular chaperones such as nucleoplasmin (73) or the recently described ATP-dependent chromatin remodeling factors (74) are specifically involved in counteracting H1-mediated repression is an open question.

In this study we have developed an in vivo system to identify H1 structural features of potential functional significance. With this system we have obtained evidence that sequences within the central globular domain are responsible for the differential effect of overproduction of two variants on gene expression. Additional mutagenesis studies will be used to further localize the sequences and structures involved towards gaining insight into how H1 interacts with chromatin to modulate gene expression.

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