The importance of histone H1 heterogeneity and total H1 stoichiometry in chromatin has been enigmatic. Here we report a detailed characterization of the chromatin structure of cells overexpressing either H10 or H1c. Nucleosome spacing was found to change during cell cycle progression, and overexpression of either variant in exponentially growing cells results in a 15-base pair increase in nucleosome repeat length. H1 histones can also assemble on chromatin and influence nucleosome spacing in the absence of DNA replication. Overexpression of H10 and, to a lesser extent, H1c results in a decreased rate of digestion of chromatin by micrococcal nuclease. Using green fluorescent protein-tagged H1 variants, we show that micrococcal nuclease-resistant chromatin is specifically enriched in the H10 variant. Overexpression of H10 results in the appearance of a unique mononucleosome species of higher mobility on nucleoprotein gels. Domain switch mutagenesis revealed that either the N-terminal tail or the central globular domain of the H10 protein could independently give rise to this unique mononucleosome species. These results in part explain the differential effects of H10 and H1c in regulating chromatin structure and function.

The fundamental repeating unit of the eukaryotic chromatin is the nucleosome, which consists of an octamer of two molecules each of the core histones H2A, H2B, H3, and H4, and in higher eukaryotes, at least a single molecule of H1 (or linker) histone (1, 2). The precise location and the number of H1 histones in the nucleosome are not known (3–6). About 166 base pairs (bp) of DNA are wrapped around the H1-containing nucleosome in two complete turns. Histone H1 is believed to make contacts with the linker DNA, and facilitate further condensation of the nucleosomal template into higher order structures such as the 30-nm chromatin fibers (7, 8).

All histones, except H4, are composed of multiple primary sequence variants (9–11) and show distinct patterns of expression during development and differentiation in a variety of organisms (9, 12–14). In the mouse, at least seven linker histone variants exist. These include the somatic variants H1a, H1b, H1c, H1d, H1e, and H10 and the testis-specific H1t variant. All these variants have the same general structure consisting of a central globular domain, a short N-terminal tail, and a longer C-terminal tail (1, 2). H1 histones have been studied in some detail and found to differ in their timing of synthesis, rates of synthesis, turnover rates, phosphorylation status, and ability to compact chromatin, but a satisfactory description of their functional significance is still missing (1, 2, 10). H10 has been termed an “extreme” somatic variant, as its sequence is considerably diverged from other somatic H1 histones (15, 16). It accumulates to high levels in non-dividing and terminally differentiated cells (17).

Over the past three decades, reports have appeared in the literature correlating the expression of particular H1 histone subtypes with changes in chromatin structure and gene expression in accordance with the developmental status of many organisms (18–21). The most dramatic effect of a H1 histone variant, histone H5, has been observed in chicken erythrocytes, where it is progressively deposited onto the chromatin as the erythrocyte matures (22, 23). This process is marked by the concomitant loss of H1 histones already bound to the chromatin, condensation of the chromatin, and an increase in nucleosome spacing. When histone H5 was inducibly overexpressed in a rat cell line, the cell cycle slowed and expression of certain genes were altered in a direct correlation with the amount of excess H5 bound to the chromatin (24). The chromatin from histone H5-overexpressing cells was found to be resistant to cleavage by micrococcal nuclease (MN) (25).

To gain an insight into the functional significance of H1 histone variants, our laboratory developed a system for the inducible overexpression of individual mouse H1 histones in cultured mouse fibroblasts (26, 27). Overexpression of individual H1 variants using this system disrupts the normal stoichiometry of the H1 variants, making it feasible to study the functional significance of individual variants in vivo. Using this system, we focused on two of the mouse H1 histone variants, H10 and H1c, and demonstrated the differential effects of their overexpression on gene expression and cell cycle progression (27). Overexpression of the H10 variant lead to reduced steady-state transcript levels for all RNA polymerase II genes studied, and a transient delay in the re-entry of G0-arrested cells into the S phase of the cell cycle following release from arrest. This is not surprising, given the similarity of histone H10 to the avian histone H5 in sequence, size, and expression patterns (15). Surprisingly, overexpression of the H1c variant led to either a dramatic increase or no change in the steady-state transcript levels of all genes tested, and had no effect on the re-entry of G0 cells into the S-phase. We further demonstrated that the differential effects of these two variants are due to differences in their globular domains (28). In this study we took advantage of the H1 variant overexpression system to see if
global differences in the structure of chromatin from cells producing either H10 or H1c could be detected and if these differences could explain the observed functional differences between these two variants.

**Experimental Procedures**

Generation of Expression Vectors and Cell Lines—The H10- and H1c-overexpressing cell lines, MTH10 and MTH1c, were described previously (26, 27). Plasmid pMTAneo was constructed by deleting the H1 coding sequences from plasmid pMT43MslAneo (27). Construction of cell lines overexpressing "domain switch" mutants of H10 and H1c have been described elsewhere (28). Plasmids pMTH10GFPAneo and pMTH1cGFPAneo were constructed using standard cloning methodology (29). Briefly, the coding sequence for the mammalian codon optimized, red-shifted, enhanced green fluorescent protein (GFP) gene was obtained from plasmid pEGFP-C1 (CLONTECH) and cloned in frame after the codon for the last lysine residue of the H10 and H1c genes obtained from plasmid pEGFP-C1 (CLONTECH) and cloned in frame after the codon for the last lysine residue of the H10 and H1c genes within the plasmids pMTH10GFPAneo and pMTH1cGFPAneo, respectively (27).

During the subcloning steps, a single alanine residue was inserted between the last lysine of the H1 proteins and the first methionine residue of the GFP protein in the H1GFP fusion proteins. Cell lines MTA, MTH10GFPA, and MTH1cGFPA were transfectected by mixing with a 20-fold excess of control nuclei, digested with MN and the combined S1 and S2 fractions were extracted with high salt buffer (0.5M KCl, 10 mM Tris-HCl, pH 7.2, 5 mM MgCl2, 0.5 mM phenylmethylsulfonyl fluoride). Insoluble material was removed by centrifugation at 16,000 × g for 5 min. Histone H1 extracted in the high salt buffer supernatant was used directly for fluorometry, as the presence of 0.5 M KCl had no effect on GFP fluorescence. Individual chromatin fractions were excited at 485 nm and the fluorescence emitted from GFP was measured at 509 nm in an Amino Bowman Series 2 luminescence spectrophotometer (Spectronics Instruments). GFP fluorescence observed in the different chromatin fractions was taken to be a measure of the relative amounts of the particular GFP-tagged H1 variant present.

**Results**

Overexpression of H1 Histone Variants Increases Nucleosome Spacing—MN digestion of chromatin from H1-overexpressing cells or control cells reveals that the vast majority of material is organized in a regularly repeating nucleosomal ladder (Fig. IA). Under these conditions H10 or H1c are about 70–75% of

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**Fig. 1.** Nucleosome spacing in cells overproducing histone H10 or H1c under exponential growth conditions. A, exponentially growing MTA, MTH10, and MTH1c cells were induced with ZnCl2 for a total of 96 h as described under "Experimental Procedures." Overexpression levels of H10 and H1c were 70% and 77% of total chromatin-bound H1 histones, respectively, as revealed by HPLC analysis. Nuclei were digested with 1.5 units/ml MN at 25 °C for 5, 10, and 15 min. DNA was isolated and electrophoresed on a 1.8% Metaphor agarose gel which was subsequently stained with EtBr. Lanes marked 100 bp contained 1 μg of a 100-bp DNA ladder (Life Technologies, Inc.). B, densitometric scans of the 10-min MN digestion patterns of MTA, MTH10, and MTH1c cell lines shown above in panel A (lanes 4–6). The x axis is the distance migrated (mm) in the gel, and the y axis is the absorbance. The arrows indicate the densitometric peak absorbance to which the size measurements for oligonucleosomes were made and correspond to mononucleosomes on the far right and oligonucleosomes of increasing size from right to left.
the total H1 species in their respective overexpressing cell line as determined by HPLC (28, 32). In addition, the total amount of H1 is estimated to be 1.2–1.4 times that of control cells (32). These results indicate that overexpression of H1 histones to high levels does not lead to the formation of aberrant chromatin structures. Overexpression of either variant does result in a clear 15–16-bp increase in nucleosome spacing from 175 bp in control MTA cells to 189 or 190 bp in the H1 histone-overexpressing cells (Fig. 1, Table I). This observed increase in nucleosome spacing agrees with results obtained with in vitro cell-free chromatin assembly systems where addition of increasing amounts of H1 histone was found to progressively increase nucleosome spacing (33–35). No increase in nucleosome spacing was observed in MTH10 and MTH1c cells not treated with ZnCl2 (data not shown).

We also investigated the effect of MN digestion of chromatin obtained from cultures induced to overexpress H1 variants under density-arrested conditions (Fig. 2A and Table I). We noted little difference among the cell lines in apparent nucleosome spacing. Upon closer inspection, we noted that the nucleosomal repeat length was close to 190 bp in all cell lines, including control cells not overexpressing H1 (Fig. 2B, compare lanes 1–3 to lanes 4–6; also see Table I). Nucleosome spacing appears to change with the cell cycle, being maximal in quiescent cultures and minimal in exponentially growing cells.

To investigate if the increase in nucleosome spacing caused by H1 histone overexpression in exponentially growing cells was coupled to DNA replication, H1 histones were overexpressed for 36 h under these conditions in the presence of 5 mM hydroxyurea (HU). Inhibition of DNA synthesis by HU was almost total, as revealed by the negligible incorporation of tritiated thymidine in these cells (data not presented). The overall amounts and percentages of overproduced H1s were slightly less due to the shorter incubation times. Nevertheless, H1 overexpression and incorporation into chromatin was observed in the absence of DNA synthesis and resulted in an increase in nucleosome spacing over control cells similar to that of exponentially growing cells in the absence of HU treatment (Fig. 2B, compare lanes 7–9 to lanes 4–6). H1 histones appear to assemble correctly on the chromatin and influence nucleosome spacing even in the absence of DNA replication (Table I).

Table I

| Cell line | Nucleosome repeat length in base pairs |
|-----------|---------------------------------------|
|           | Density-arrested cells | Exponentially growing cells | Exponentially growing cells in hydroxyurea |
| MTA       | 188                     | 175                     | 177                     |
| MTH10     | 192                     | 190                     | 189                     |
| MTH1c     | 191                     | 189                     | 188                     |

In vivo assay of nucleosome spacing in cell lines overexpressing histone H1 variants

Nucleosome repeat lengths in MTA, MTH10, and MTH1c cell lines are shown. Photographic negatives of gels similar to those shown in Figs. 1A, 2A, and 2B were scanned on a densitometer. Nucleosome spacing for each cell line was determined by comparing the mobilities of bands corresponding to tri- through hexa-nucleosomes to the standard curve generated for the migration of individual bands in the 100-bp ladder, as previously described (48). Average values from three independent experiments are shown.
were 69% and 77%, respectively. Overexpression levels of 
H10 and H1c cells overproducing H10 or H1c under conditions of exponential growth or density arrest were 74% and 85%, respectively. (data not shown). To extend this observation, we designed an experiment to determine the effect of H1 histone variants on chromatin structure in vivo. For this assay, we used stable cell lines that express the chimeric H1 variant-GFP constructs H10GFP (cell line MTH10GFP) or H1cGFP (cell line MTH1cGFP). These cell lines express low levels of the H1GFP fusion proteins (5–7% of the total H1 histone present), and do not significantly perturb the natural stoichiometry of the H1 variants. GFP fluorescence from these chimeric proteins allows us to easily compare the amounts of individual H1 variants in the MN-soluble and -insoluble chromatin fractions. These H1GFP fusion proteins behave identically to their respective native parent H1 variants in that they are localized specifically to the chromatin in the nucleus, give rise to regular MN digestion and NP patterns (see below), and appear to bind chromatin with the same affinity as the parent H1 variants (data not shown). Because of the low levels of expression of the H1GFP fusion proteins, the rates of MN digestion of chromatin from these lines were identical to control MTA cells. The amount of H10GFP and H1cGFP present in the MN-soluble and -insoluble chromatin fractions obtained from density-arrested cells was quantitated by fluorometry (Fig. 4). After 15 min of digestion with MN, 77% of H10GFP was retained in the MN-resistant chromatin pellet compared with only 34% of H1cGFP.

**The Mono- and Dinucleosome Banding Patterns of Chromatin from H10- and H1c-overexpressing Cells Are Different**—To carry out a more detailed structural characterization of the chromatin from H10- and H1c-overexpressing cells, we employed the technique of composite NP gels (31). These high resolution agarase-polyacrylamide-glycerol gels can resolve individual mono-, di-, and higher order nucleosomes based on the conformation of these particles, the length of DNA, and the number of histone H1 and HMG molecules associated with them. When MN-soluble S2 chromatin was run on these gels, five bands (M1 through M5) corresponding to mononucleosomes and three bands (D1 through D3) corresponding to dinucleosomes were resolved (Fig. 5A). This banding pattern com-
Effects of H1 Histones on Chromatin Structure

FIG. 5. NP patterns of mono- and dinucleosomes obtained from the chromatin of cells overexpressing histone H10 or H1c. Nuclei were isolated from density-arrested MTA, MTH10, and MTH1c cells following induction with ZnCl2, and digested with 1.5 units/ml MN for 15 min at 25 °C. Overexpression levels of H10 and H1c were 77% and 85% of total chromatin-bound H1 histones, respectively. A, the MN-soluble S2 chromatin fraction from control and H1 variant-overexpressing cells was resolved on composite NP gels as described under “Experimental Procedures,” and the NP pattern was visualized by EtBr staining. The unique faster migrating M III mononucleosome species observed upon overexpression of H10 is indicated by the arrow. B, the mononucleosome bands from the M I, M II, and M III bands were excised and total histones were extracted as described previously (31). Aliquots were run on 17% polyacrylamide-SDS gels, and histones were visualized by staining with Coomassie Blue.

FIG. 6. Direct visualization and identification of mono- and oligonucleosomes carrying bound H1 histones using GFP-tagged H10 and H1c on NP gels. Density-arrested MTH10GFP and MTH1cGFP cells were induced with 100 μM ZnCl2 as described under “Experimental Procedures.” Overexpression levels of H10GFP and H1cGFP were 19% and 13% of total chromatin-bound H1 histones, respectively. Nuclei were isolated and digested with 1.5 units/ml MN for 15 min at 25 °C. MN-soluble S2 chromatin was run on NP gels as described under “Experimental Procedures.” The gels scanned on a Molecular Dynamics Storm fluorimaging unit in the blue fluorescence mode to visualize the GFP fluorescence from NP bands carrying bound H1GFP fusion proteins and then stained with EtBr to visualize all the NP bands. Note that bleed-through of GFP fluorescence from bands carrying H1GFP fusions contributes to the fluorescent signal observed in the lanes marked EtBr. The lane labeled MTA contains material from control cells that do not express H1-GFP hybrids.

The NP patterns of chromatin from cells expressing the H1GFP fusion proteins was also analyzed. Fluorescence from GFP allows direct visualization and identification of nucleosome species containing bound H1GFP. Images showing GFP and EtBr fluorescence from the same gel are shown for both H10GFP- and H1cGFP-expressing cell lines (Fig. 6). The major mononucleosome bands containing H1GFP fluorescence probably represent M III-like species, which, due to the additional mass of the GFP moiety (~28 kDa), migrate more slowly on NP gels. Interestingly, this band migrates faster in H10GFP samples than in H1cGFP samples, suggesting a more compact nucleosomal conformation.

Both the N-terminal Tail and the Central Globular Domain of the H10 Protein Are Independently Capable of Giving Rise to
FIG. 7. NP patterns of mono- and dinucleosomes obtained from the chromatin of cells overexpressing histone H10, H1c, and their “domain switch” mutants. Density-arrested cultures of the indicated cell lines were induced with 100 μM ZnCl2. Hybrids are designated by the variant from which the N-terminal, globular, and C-terminal domains were derived, i.e., 00C is composed of the N-terminal and globular domains of H10 and the C-terminal domain of H1c. MTH1C0Cdel carries a small deletion in the C-terminal tail. Nuclei were prepared, and the MN-soluble chromatin was resolved on composite NP gels as described under “Experimental Procedures.” The NP pattern was visualized by EtBr staining. Overexpression levels were as follows: H10 = 78%; H1C0Cdel = 80%; H100C = 77%; H1C00 = 74%; H1C0 = 81%; H10CC = 78%; H1CC0 = 65%; H1c = 86%.

Effects of H1 Histones on Chromatin Structure

The role played by linker histones in regulating chromatin structure and gene expression is controversial (4, 40–42). Early studies, with a few notable exceptions (24, 25, 43), were unable to show a clear and direct correlation between individual H1 variants, chromatin structure and chromatin function. We developed a system for overexpressing individual H1 genes in homologous mouse cells, thereby perturbing the normal stoichiometry of variants (26, 27). Initially, we showed that overexpression of H10 and H1c variants lead to different effects on gene expression and cell cycle progression, the former having an inhibitory effect, and the latter having either a stimulatory effect or no effect at all (27). We also demonstrated that the differential effects of H10 and H1c on gene expression are due to differences in the central globular domains of these two proteins (28).

In this study we demonstrated alterations in chromatin structure upon the overexpression of H10 and H1c. Some of the alterations were variant-specific, while others occurred upon overproduction of either variant. The latter class includes an increase in nucleosome spacing in exponentially dividing cells, increased MN resistance in density-arrested cells, and increased levels of slow migrating dinucleosome species in NP gels. These effects may be due to increased levels of total H1 per nucleosome associated with overproduction of either variant. Several reports have argued that two or more H1 histones can bind per nucleosome (5, 6, 44, 45). Whether these “extra” H1 histones bind to these nucleosomes with the same affinity as the first H1 is not known. Also, it is not clear whether the extra H1 histones associate with the core nucleosome in the same manner as the initial H1, or mainly with the linker DNA with an affinity equal to the its affinity for naked DNA (6). The binding of more than one H1 histone per nucleosome is likely to play important roles in gene regulation, especially if this occurs in only a subset of the chromatin.

We also observed alterations in chromatin structure that were associated specifically with the overexpression of the H10 variant. These include increased MN-resistance of chromatin from exponentially growing cells, preferential distribution of H10 in MN-resistant chromatin fractions, and the presence of a unique faster migrating mononucleosome species in NP gels. In light of our earlier studies involving the effects of H1 variant overexpression on gene expression and cell cycle progression (27), we propose a simple, coherent model for the differential effects of H10 and H1c variants.

We suggest that histone H10 functions as a “replacement variant” (46) in vivo and replaces other H1 variants in G2-arrested cells. H10 may then result in the progressive increase in condensed chromatin in quiescent cells. This is supported by the observation that H10 accumulates naturally in non-dividing, terminally differentiated cells, which exhibit compact chromatin, along with reduced levels of transcription and no replication (17).

Overexpression of H10 during any phase of the cell cycle simply mimics the natural accumulation of this variant in growth-arrested cells, leading to the formation of condensed chromatin. Due to the inaccessibility of binding sites for both the chromatin of cells overexpressing histone H10, H1c, and their “domain switch” mutants. Density-arrested cultures of the indicated cell lines were induced, designated by the variant from which the N-terminal, globular, and C-terminal domains were derived, i.e., 00C is composed of the N-terminal and globular domains of H10 and the C-terminal domain of H1c. MTH1C0Cdel carries a small deletion in the C-terminal tail. Nuclei were prepared, and the MN-soluble chromatin was resolved on composite NP gels as described under “Experimental Procedures.” The NP pattern was visualized by EtBr staining. Overexpression levels were as follows: H10 = 78%; H1C0Cdel = 80%; H100C = 77%; H1C00 = 74%; H1C0 = 81%; H10CC = 78%; H1CC0 = 65%; H1c = 86%.

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2 A. Gunjan, B. T. Alexander, D. B. Sittman, and D. T. Brown, unpublished observations.
the basal transcriptional machinery and specific transcription factors within this condensed chromatin, there is a general repression of gene expression, as observed in our earlier studies (27). Compensatory mechanisms probably help maintain a minimal level of gene expression, to assure survival of the cell. The natural accumulation of H1\(^0\) in quiescent cells is mediated in part by the replication-independent mode of expression of the endogenous gene (17). However, properties of the H1\(^0\) protein, most notably the preferential association with MN-resistant, presumably condensed (47), chromatin observed in this study may also contribute to the accumulation of this variant and its proposed role in stabilizing the quiescent state.

As shown in our earlier studies, histone H1c is functionally opposed to H1\(^1\) (27, 28). Histone H1c appears to be deficient in condensed chromatin when compared with H1\(^0\). Overexpression of H1c to high levels in exponential cells does not lead to a very high degree of chromatin compaction as assayed by its sensitivity to MN cleavage (Fig. 3A). H1c therefore appears to be associated in vivo with chromatin regions that have a relatively "open" architecture (37, 38), which might facilitate transcription and replication by allowing easy access to the factors involved in these processes. Overexpression of H1c might accentuate the normal functions of H1c by making larger quantities of this protein available for binding to regions not normally occupied by this variant. This would "open up" extensive regions of the chromatin, making it readily accessible for the binding of trans-activating factors and leading to the enhanced expression of some genes, as demonstrated in our previous studies (27).

The identification that both the N-terminal tail and the central globular domain of the H1\(^0\) protein can independently give rise to the unique high mobility M III mononucleosome species is interesting (Fig. 7). The involvement of the globular domain of H1\(^0\) in the formation of the high mobility M III species was to be expected, based on our earlier results on the effects of this domain on gene expression (28). That the short N-terminal tail of H1\(^0\) also affects mononucleosome mobility, possibly by affecting its conformation, implies that the tails of the H1 proteins may play important roles in some of their functions.

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