MENT, a Heterochromatin Protein That Mediates Higher Order Chromatin Folding, Is a New Serpin Family Member*

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Terminal cell differentiation is correlated with the extensive sequestering of previously active genes into compact transcriptionally inert heterochromatin. In vertebrate blood cells, these changes can be traced to the accumulation of a developmentally regulated heterochromatin protein, MENT. Cryoelectron microscopy of chicken granulocyte chromatin, which is highly enriched with MENT, reveals exceptionally compact polynucleosomes, which maintain a level of higher order folding above that imposed by linker histones. The amino acid sequence of MENT reveals a close structural relationship with serpins, a large family of proteins known for their ability to undergo dramatic conformational transitions. Conservation of the “hinge region” consensus in MENT indicates that this ability is retained by the protein. MENT is distinguished from the other serpins by being a basic protein, containing several positively charged surface clusters, which are likely to be involved in ionic interactions with DNA. One of the positively charged domains bears a significant similarity to the chromatin binding region of nuclear lamina proteins and with the AT-rich DNA-binding motif, which may account for the targeting of MENT to peripheral heterochromatin. MENT ectopically expressed in a mammalian cell line is transported into nuclei and is associated with intranuclear foci of condensed chromatin.

In eukaryotic cells, DNA in association with histones and other nuclear proteins forms a DNA-protein complex or chromatin that exhibits varying levels of compaction (1, 2). Chromatin is folded hierarchically, with the basic level represented by a repeated structural unit, the nucleosome, comprising 200 ± 40-bp DNA of which 146 bp make about 1.7 superhelical turns around the histone octamer (3), and the remainder (linker DNA) is not constrained by core histones. Linker DNA is usually associated with the ninth (linker) histone, which brings the core-proximal segments of linker DNA together (4–6). In vitro, nucleosome arrays fold into ~30-nm-wide chromatin filaments having a characteristic zigzag organization at low ionic strength with separate nucleosomes connected by extended linkers (7–10). If the extent of DNA charge shielding or neutralization is increased (e.g. by increasing the salt concentration in the medium), the zigzag chromatin fibers first become more compact and then self-associate to form the next level of higher order folding, which involves closer contacts between nucleosomes (11–15). Modulation of chromatin folding constitutes a potentially important regulatory mechanism that may influence the locus-specific accessibility of DNA templates to the trans-acting factors mediating transcription (16–19), replication (20), transposition (21), and perhaps other template-dependent functions.

It has been known for many years that, in interphase nuclei, the chromatin is not uniformly decondensed but contains a considerable amount of more compact material, called heterochromatin (22), which generally increases during the terminal stages of eukaryotic cell differentiation (23). Much cytological and genetic evidence suggests that chromatin compaction (spreading of heterochromatin) is associated with position-specific genetic inactivation (24–28). A surprisingly high diversity of structurally distinct heterochromatin-associated proteins has been reported. For example, SIR3, SIR4, and RAP1 proteins are involved in telomeric silencing in Saccharomyces cerevisiae (29, 30), Pdd1p is involved in chromatin condensation and elimination in Tetrahymena thermophila (31), and the numerous Su(var) and Polycomb Group proteins of Drosophila melanogaster (26, 32, 33) are implicated in chromosomal locus-specific genetic repression mediated by chromatin structure, as are the related “chromo” domain-containing vertebrate proteins (34, 35).

Although several studies have indicated that stable alterations of chromatin higher order folding distinguish the organization of euchromatin and heterochromatin (13, 36, 37), the structural determinants and the molecular mechanism of heterochromatin formation and spreading are still obscure. Terminal cell differentiation provides a convenient system for biochemical studies of the coordinated formation of heterochromatin and associated genetic inactivation. In particular, blood cell differentiation or hemopoiesis has been extensively studied both for its applicability to gene regulation and for its intrinsic significance for oncology (38, 39). In the normal developmental sequence, the number of expressed genes becomes drastically reduced (40, 41), the nuclear chromatin undergoes a considerable increase in condensation (see reviews in Ref. 42), and nuclear matrix components, which are associated with transcriptional activity, are progressively lost (43).

Based on the hypothesis that the extensive heterochromatin spreading in blood cells is caused by one or more of the relatively few proteins that are still expressed during terminal differentiation, we have sought highly abundant nuclear proteins that specifically interact with repressed chromatin and are confined to the late stages of cell differentiation. Our study has yielded an abundant developmentally regulated 42-kDa

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† The abbreviations used are: bp, base pair(s); PBS, phosphate-buffered saline; MNase, micrococcal nuclease; PCR, polymerase chain reaction; ORF, open reading frame; NLS, nuclear localization signal.
nuclear protein, MENT (gyreloid and erythroid nuclear termination stage-specific protein). This protein is expressed in terminally differentiated blood cells, is associated with repressed chromatin, and is able to induce large scale condensation of nuclear chromatin and the dissociation of inactivated chromatin from the nuclear matrix in vitro (44, 45). MENT is present in all three main avian blood cell types (erythrocytes, lymphocytes, and granulocytes) and is especially abundant in granulocytes, where it becomes the predominant nuclear nonhistone protein (~2 molecules/nucleosome) and is concentrated in the compact peripheral heterochromatin (46). MENT-like polypeptides have also been found in chromatin of mammalian leukocytes.2

We have isolated the compact granulocyte chromatin in a soluble form and applied croyelectron microscopy, a powerful imaging technique permitting the visualizing of the native organization of unfixed biological material (47) to the study of heterochromatin conformation. For the first time, we have documented a direct link between heterochromatin and higher order folding of nucleosome arrays that is attributed to the accumulation of a single nonhistone protein, MENT, in chromatin fibers. We have cloned the MENT cDNA, deduced the protein primary structure, and identified the protein structural motifs that may account for the molecular interactions of MENT. The combined results suggest a molecular basis for the formation of MENT-directed heterochromatin.

MATERIALS AND METHODS

Cells, Nuclei, and Chromatin—Polygramnuclear granulocytes were isolated from peripheral blood of adult white leghorn chicken as described (46). COS-7 cells (CV-1 simian cells transformed with origin-defective SV-40 virus expressing wild-type T antigen, ATCC number CRL-1651) were grown to 80% confluency in Dulbecco's modified Eagle's medium (D-5796; Sigma) containing 10% fetal calf serum (F-20442; Sigma) and 1 mM pyruvate. Cell cultures were washed 2 times in PBS containing 0.14 NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.7 mM KH2PO4, pH 7.5, and the cells were resuspended in PBS using a cell scraper. To isolate the cells, suspension of granulocytes or COS-7 cells in PBS were centrifuged for 3 min at 1000 x g and resuspended in reticulocyte standard buffer containing 10 mM NaCl, 3 mM MgCl2, 10 mM Tris-HCl, pH 7.5, plus 0.5% Nonidet P-40 (Nonidet P-40; Life Technologies, Inc.) and 1 mM phenylmethylsulfonyl fluoride. With granulocyte preparations, the NaCl concentration varied between 0 and 0.3 M. The cell suspensions were homogenized by 20–30 strokes of pestle A in a Dounce homogenizer over 30 min on ice. Nuclei were centrifuged for 10 min at 7600 x g, and the nuclear pellets were resuspended in reticulocyte standard buffer plus 1 mM phenylmethylsulfonyl fluoride. Isolated nuclei could be stored for a week at +2°C without a detectable DNA or protein degradation.

Micrococcal nuclease (MNase) digestion of all types of nuclei was conducted as described (46) and terminated by adding EDTA to 10 mM. To obtain soluble chromatin, the nuclei were digested with MNase to obtain an average DNA fragment size between 400 and 6000 bp and centrifuged for 5 min at 10,000 x g. The supernatant S1 was removed, and the pellet was resuspended in 1 ml of TEN (Tris-EDTA-NaCl buffer containing 0.14 NaCl, pH 7.5, 1 mM EDTA, 10 mM NaCl). Supernatant S2 containing more than 50% of the input nuclear chromatin was then obtained after centrifugation for 5 min at 10,000 x g. The nuclear pellets were washed once more with 1 ml of TEN buffer with centrifugation to provide the S3 supernatant, and the nuclear remnant fraction was obtained by dissolving the final pellet in 1 ml of 0.5% SDS.

Electrophoretic Techniques—DNA electrophoresis in agarose, polyacrylamide gel electrophoresis of proteins, detection of proteins and nucleic acids, Western blotting, probing with anti-MENT antibodies, and quantitative densitometry of electrophoregrams were conducted as described (46). Protein to DNA ratios were estimated from parallel measurements of DNA concentration by UV spectrophotometry (A260 = 1 for 50 μg/ml DNA).

Ultracentrifugation—For size fractionation of soluble chromatin, 0.5 ml of S-150 sample containing 0.2 mg/ml DNA was loaded on a 5–25% sucrose gradient (12 ml) containing TAEW (25 mM sodium acetate, 2 mM Na2-EDTA, 20 mM Tris acetate, pH 7.4) or HEN (HEPES-EDTA-NaCl buffer containing 40 mM NaCl, 1 mM EDTA, 10 mM HEPES, pH 7.5). Ultracentrifugation was carried out in an SW-41 rotor on a Beckman L-8–50 ultracentrifuge for 3 h at 4 °C and 35,000 rpm. 1-ml fractions were collected and used for protein and DNA electrophoreses. The final pellet fraction (fraction 1) was resuspended from the bottom of the tube in 1 ml of 0.5% SDS.

Cryoelectron Microscopy—Cryoelectron microscopy using soluble chromatin samples (50–100 μg/ml in TEN buffer) was conducted as described (5, 48). Specifically, 3-μl chromatin samples were applied to holey carbon films, blotted with Whatman 52 filter paper, and plumped into liquid ethane held just above its freezing point in liquid nitrogen. Grids were transferred under liquid nitrogen to a cryoholder (model 626; Gatan Inc., Pleasanton, CA), and observed at ~170 °C in an electron microscope (CM10; Philips Electronic Instruments Co., Mahwah, NJ) at a nominal magnification of × 45,000. Tilt pairs of micrographs (angular separation 30°) at 1.1–1.5-μm defocus, were recorded in low dose mode, on film 50–163 (Eastman Kodak Co., Rochester, NY) and developed in full-strength D-19 (Kodak) for 12 min.

Cloning and Sequencing of MENT cDNA—MENT protein was isolated from the nuclei of unfractionated chicken blood cells as described (44). Five peptides derived from isolated MENT were sequenced. For PCR-mediated cloning of MENT cDNA, we designed redundant oligonucleotide primers as described (49). The 20–22-nucleotide-long primers were designed from N-terminus regions with amino-terminal codons. Total RNA and poly(A)+ mRNA was isolated from 1 g of chicken bone marrow using Trizol reagent (Life Technologies, Inc.) and the Message Maker RNA isolation kit (Life Technologies), and the first strand cDNA synthesis was conducted using the Life Technologies Superscript preamplification system essentially as described in the vendor's manual. PCR amplifications with degenerate primers were carried out using the AmpliTherm polymerase and MasterAmp PCR optimization kit (Epicentre Technologies, Madison, WI). Amplification cycles were carried out in 30-μl samples using a high melting wax for “hot start” conditions. The first denaturation for 5 min at 94 °C was followed with 35 cycles, each containing three 1-min steps at 94, 42, and 72 °C, and finally for 10 min at 72 °C. PCR products were cloned into pCRII vector (Invitrogen) and sequenced using the Sequenase II system (Amersham Pharmacia Biotech). One of the sequenced PCR fragments amplified between the primers specific for peptides 1 (ATIGGIAA(C/T)-TT(T)/CTACIGTIGA) and 3 (TGIATAA/G(TT)/CT(T)/TCG(C/T)/TG(T)-CT) also included the sequence of peptide 2, indicating that this PCR product was derived from the target MENT cDNA.

To restore the 5′-end of the cDNA, we employed the 5′-rapid amplification of cDNA ends PCR technique (50) essentially as described (51). The 5′-end of the cDNA was restored using the 3′-rapid amplification of cDNA ends PCR method (50). The PCR-amplified DNA fragments were inserted into pCRII vector (Invitrogen) and sequenced on both strands, revealing a 1230-bp open reading frame (ORF) encoding all five independently sequenced MENT peptides. To verify the ORF sequence, PCR amplification was repeated with two nonredundant primers flanking the ORF, and the product was inserted in the pCRII vector and sequenced on both strands with the Sequenase II system.

cDNA Expression and Cell Immunofluorescence—MENT ORF was amplified by reverse transcription-PCR with oligonucleotide primers containing appropriate restriction sites and inserted in pReCMV vector (Invitrogen) to provide a MENT-expressing plasmid, pSG109. COS-7 cells were transfected with pSG109 and Supersfect transfection media (Qiagen) as described in the vendor's manual. Transfected cells were grown for 48 h. Fixing of cells attached to the cover glasses with methanol/acetone and staining with anti-MENT antibodies and DNA dyes (10 μg/ml Hoechst 33258) was performed essentially as described (45). For double-staining with anti-MENT and anti-lamin B antibodies (52) antibodies, cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Fluorescence and confocal microscopy were conducted as described (45).

Protein Sequence Analysis and Three-dimensional Modeling—A search for DNA and protein homologies in the available data banks was performed using the BLAST program (53). Sequence alignments were conducted using the Wisconsin package version 9.1 (Genetics Computer Group, Madison, WI). The protein pl was calculated as described (54). To build the three-dimensional protein model, we employed the ProMod-II software (55, 56). The model-building resources are provided by the automated protein modeling server, Swiss Model (Glaxo Wellcome Experimental Research, Geneva, Switzerland), which is accessible through the Internet. The protein models were visualized using the Swiss-PDB viewer program compatible with Windows 95™. Modeling of electrostatic and surface properties of the molecules was performed.

S. Grigoryev, unpublished observations.

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RESULTS

Solubilization of Tightly Packed Granulocyte Chromatin—Highly condensed chromatin from chicken granulocyte nuclei has a nucleosome repeat \((192 \pm 2 \text{ bp})\) and a core and linker histone content \((46)\) similar to that of many cells with much more active genomes \((1)\). Unlike most other types of chromatin, which are readily solubilized after MNase digestion and removal of divalent cations, granulocyte nuclei digested to DNA fragment sizes between 200 and 20,000 bp did not release any soluble chromatin after repeated washing in low salt/high pH media. Our previous work suggested that MENT, an extremely abundant granulocyte chromatin protein \((2.1 \text{ molecules/nucleosome})\) was the most likely factor inhibiting chromatin solubility \((46)\). To address the impact of auxiliary proteins on chromatin conformation, it was essential to isolate granulocyte chromatin from the nuclei while maintaining its association with MENT. We therefore determined the optimal salt concentration allowing chromatin solubilization with a minimal loss of associated chromatin proteins by preparing a series of granulocyte chromatin fractions eluted from nuclease-digested nuclei with varying concentrations of NaCl. An abrupt increase in chromatin solubility occurred between 100 and 150 mM NaCl (Fig. 1, a and b). The amount of MENT retained in chromatin also changed very considerably between these two NaCl concentrations, as shown by Western blotting of nuclear proteins probed with anti-MENT antibodies (Fig. 1c).

Granulocyte nuclei washed with 150 and 300 mM NaCl (samples CG-150 and CG-300) retained, respectively, 25 and 1.4% of the original MENT and were used for isolating soluble chicken granulocyte chromatin after limited MNase digestion. Further characterization was carried out on the soluble chromatin fraction S2 (see “Materials and Methods”), which contained about 50–90% of total nuclear DNA. The MENT/DNA ratio was the same in the TEN-washed nuclei as in the soluble chromatin fractions. Depending on the extent of micrococcal nuclease digestion, the average size of nucleosome chains \(N_{\text{av}}\) in S2 preparations varied between 3 and 50 without affecting the protein composition of the solubilized material (data not shown).

MENT Is Integrated into Soluble Polynucleosome Arrays—Since the isolation of granulocyte chromatin required a considerable depletion of MENT, it was important to determine if the residual protein was bound firmly enough to be considered as an integrated architectural element of soluble polynucleosomes. We subjected soluble granulocyte polynucleosomes (CG-150, \(N_{\text{av}} = 20\)) to ultracentrifugation in 5–25% sucrose gradients containing 50 mM monovalent ions and analyzed the gradient fractions by DNA electrophoresis and Western blotting (Fig. 2, a and b). All input MENT cosediments with chromatin (Fig. 2b), none being found at the top of the gradient. Thus, the behavior of MENT is in sharp contrast to the ubiquitous architectural nonhistone chromatin protein, HMG-1, most of which does not co-sediment with chromatin under these conditions \((58)\).

We observed a significant enrichment in MENT \((0.84 \text{ molecules of MENT/nucleosome})\) in the fastest sedimenting fraction containing long (>20-mer) polynucleosomes. Small oligonucleosomes contained less MENT, with practically none recovered from the mononucleosome fraction. As discussed below, the distribution of MENT is consistent with the greater compaction of long polynucleosomes (Fig. 3, a–f).

The low level of MENT in short oligonucleosomes appears to reflect their origin in less condensed “euchromatin” rather than their inability to bind the protein; when we ran a sucrose gradient of a granulocyte chromatin sample from the same nuclei (CG-150) but obtained after extensive cleavage by MNase \((N_{\text{av}} = 3)\), MENT was abundant in both the oligonucleosome and mononucleosome fractions, and again no free protein was detected (data not shown). Therefore, the most likely explanation for the preferential association of MENT with long polynucleosomes is that this fraction originated from heterochromatic nuclear domains relatively protected from nuclease digestion (MENT inhibits MNase digestion and is unevenly distributed in granulocyte nuclei, being preferentially associated with compact heterochromatic areas \((46)\)). As the heterochromatin becomes more extensively digested, MENT then appears in the oligonucleosome and mononucleosome fractions.

To determine whether any other proteins besides MENT are associated with compact granulocyte chromatin, we analyzed the protein composition of nuclear and soluble chromatin released after the 150 mM NaCl treatment (Fig. 2c, lanes 1 and 2) and after sedimentation of oligonucleosomes \((N_{\text{av}} = 3)\) through a sucrose gradient (lane 3). Densitometry of the Coomassie-stained gel shows that soluble CG-150 chromatin (lane 2) retains 26% of the MENT present in untreated granulocyte nuclei (lane 1), which, from the previous estimation of 2.1 MENT molecules/nucleosome in granulocytes \((46)\), indicates that about 0.5 molecules of MENT/nucleosome are associated with
Heterochromatin Formation by MENT

Fig. 2. MENT is firmly associated with soluble granulocyte chromatin. a, agarose electrophoresis of DNA from sucrose gradient fractions 1–13 obtained after ultracentrifugation of long granulocyte chromatin \(N_{av} = 20\) in HEN buffer (ethidium bromide stain). b, Western blotting of the material isolated from combined sucrose gradient fractions: 1 (lane 1), 2–4 (lane 2), 5–7 (lane 3), 8–10 (lane 4), and 11–13 (lane 5). Detection with anti-MENT antibodies. c, SDS-polyacrylamide gel electrophoresis of proteins from total granulocyte nuclei (lane 1); soluble granulocyte chromatin, CG-150 (lane 2); and the oligonucleosome pellet obtained after ultracentrifugation of CG-150, \(N_{av} = 3\) (lane 3) (Coomassie R-250 stain).

Total soluble chromatin. After centrifugation, MENT remains the only prominent band among the nonhistone proteins (Fig. 2c, lane 3). All other abundant nonhistone proteins such as actin and Mim-1 present in the crude nuclear preparation \(46\) were lost during sedimentation, indicating that they have a low affinity for chromatin. The core and linker histone content is typical of somatic nuclei, with three major subfractions of histone H1 giving a total of \(\sim 1\) linker histone/nucleosome in all chromatin fractions (Fig. 2c), demonstrating that MENT is added to, but does not replace, other histones in granulocyte nuclei. In contrast, heterochromatin in mature avian erythrocytes contains \(\sim 0.5\) H1 molecules/nucleosome, the H1 being partially replaced by H5 during development \(1\).

We thus conclude that MENT is the single protein in granulocyte chromatin whose abundance and distribution in chromatin is consistent with it being a principal factor in the extensive heterochromatization that occurs in these cells. In native granulocyte nuclei, this protein is present at a concentration that renders the chromatin completely insoluble; by removing 75% of the nuclear MENT, we were able to isolate a chromatin fraction suitable for biochemical and structural analysis.

MENT-associated Granulocyte Polynucleosomes Retain a Compact Higher Order Folding in Low Ionic Strength Media—Cryoelectron microscopy has recently emerged as a powerful technique allowing visualization of the solution conformation of biological material suspended in the buffer of choice. The three-dimensional configuration can be recovered, and the common artifacts associated with fixation, staining, and flattening inherent in standard transmission electron microscopy techniques are avoided \(47, 48\). This technique has been used to obtain a detailed characterization of the three-dimensional zigzag conformation of polynucleosomes in low salt media \(5\).

For cryoelectron microscopy, we used the long chromatin \(N_{av} = 20\) from granulocyte nuclei, which retained about 0.5 molecules of MENT/nucleosome (Fig. 1c). When vitrified in 20 mM monovalent ions, these granulocyte polynucleosomes (Fig. 3, a–d) display a considerably more compact structure than do other types of chromatin under similar conditions (Fig. 3, j–l; see also Refs. 5 and 59), appearing as compact fibers ranging in diameter from 30 to 50 nm. Nucleosome disks are seen predominantly at the periphery of the compact fibers and do not form close contacts with each other. When visible, the entry/exit sites of the linker DNA segments are always oriented toward the fiber interior. The arrangement of nucleosomes in the compact chromatin fibers is nonuniform, with no evidence of symmetry or a helical architecture, but is consistent with higher order folding based on a three-dimensional zigzag that allows a high degree of nonuniformity of the structure \(9\).

Two novel features were seen in the long compact chromatin. First, some fibers showed a backbone-like axial structure that runs perpendicular to the DNA linker segments and appears to be located toward the center of the fiber (Fig. 3, b and c, arrows). The axial structures, which were also seen by conventional transmission electron microscopy of fixed granulocyte oligonucleosomes (not shown) but never observed in MENT-depleted, decondensed chromatin, may result from a juxtaposition of linker DNA segments induced by MENT. Since DNA gives much more contrast than protein in cryoelectron microscopy \(5\), it is unlikely that the axial structures represent MENT itself. Another feature of MENT-containing condensed chromatin is its tendency to form, in addition to “30-nm” fibers (Fig. 3, b and d), thicker and more electron-dense structures (Fig. 3, a and c). In some cases, these appear to originate from the folding of a chromatin fiber back on itself (Fig. 3c).

The small granulocyte oligonucleosomes (<10-mers) observed in the same preparations are considerably more unfolded (Fig. 3, e–i) and are closer in appearance to the typical low salt conformation observed with other chromatin types (Fig. 3, j–l; see also Ref. 5). The more relaxed conformation of the small oligonucleosomes is consistent with the differential distribution of MENT, which has a strong preference for longer chromatin fragments and is practically absent from small particles (Fig. 2, a and b).
Although preferential binding of MENT to longer polynucleosomes might involve protein redistribution similar to that reported for linker histones (11, 60), other data suggest that the observed images are not due to this potential artifact. For example, when we reassociated MENT with soluble polynucleosomes, we observed compact but irregular structures without any signs of side-by-side fiber organization (46).

As another control, we studied the solution structure of “euchromatin” derived from actively proliferating COS-7 cells. In the same 20 mM ionic strength buffer, this chromatin (Nerv ≈ 20) had a typical open zigzag organization (Fig. 3, j–l) similar to that observed with chicken erythrocyte chromatin at low salt (5). Even in a 40 mM ionic strength buffer, the compaction of COS-7 nucleosome fibers was lower than with MENT-containing granulocyte chromatin at 20 mM (not shown). Thus, under similar ionic conditions, the two types of chromatin display striking differences in the extent of compaction.

MENT cDNA, Primary Structure, and Similarities to Other Proteins—We used information derived from the partial amino acid sequence of five peptides isolated from purified MENT to design redundant oligonucleotide primers and to amplify a MENT cDNA clone from chicken bone marrow poly(A)+ mRNA (see “Materials and Methods”). The cDNA clone obtained contained a 2074-bp nucleotide sequence including a 1230-bp ORF, which encoded a 410-amino acid polypeptide. These sequence data have been submitted to the GenBank™ data base under accession number AF053401. The protein sequence deduced from the ORF contained the sequences of all five independently isolated peptides (the underlined portions of the MENT sequence in Fig. 4a). The first ATG codon in the ORF was also found at the start of one of the peptides, showing the first ATG to be a probable translation initiation codon. The first nonsense codon of the 1230-bp ORF was also the limit of serpin protein homology (see below), apparently marking the C terminus of the protein. The predicted molecular mass of the 410-amino acid protein (47,383 Da) is slightly higher that the molecular mass deduced from SDS-polyacrylamide gel electrophoresis of MENT (42 kDa), further confirming that the full sequence of MENT has been revealed.

The MENT ORF had no exact matches to other proteins in the available data bases but revealed a highly significant sequence homology to a number of proteins belonging to the ovalbumin subfamily of serpin proteins (Fig. 4a). The similarity with serpins included most of the MENT sequence. Although among serpins there are a number of proteins that fulfill different noninhibitory functions (61, 62), including the recently discovered nuclease activity of leukocyte elastase inhibitor in apoptosis (63), as far as we know, none has previously been...
shown to be a chromatin-associated nuclear protein. MENT thus appears to be a novel chromatin-binding structural protein.

Like ovalbumin, MENT does not inhibit proteases (46), and its "reactive center" peptide bond, T–T, does not resemble reactive centers of any of the protease-inhibiting serpins (the triangle in Fig. 4a shows the standard position of the reactive site P1 residues in the active inhibitors: bomapin and horse leukocyte elastase inhibitor. The asterisks show the position of the putative NLS in MENT. The peptide sequences determined by direct protein sequencing are underlined. The M-loop domain (positions 69–91) is marked by a solid box, and the "hinge" motif (positions 357–364) is marked by caret marks.

**Fig. 4.** Amino acid sequence of MENT and comparison with other proteins. a, alignment of the amino acid sequence of MENT deduced from its cDNA with those of the related serpins: human bomapin (accession no. P48595), horse leukocyte elastase inhibitor (hlei; accession no. P05619), and chicken ovalbumin (oval; accession no. P01012). Unshaded areas show the MENT sequence and identical amino acid residues in other proteins. The triangles indicate the reactive site P1 residues in the active inhibitors: bomapin and horse leukocyte elastase inhibitor. The asterisks show the position of the putative NLS in MENT. The peptide sequences determined by direct protein sequencing are underlined. The M-loop domain (positions 69–91) is marked by a solid box, and the "hinge" motif (positions 357–364) is marked by caret marks. Vertical lines and colons show identities and similarities between each of the lamin protein sequences and MENT. b, comparison of the amino acid sequences of MENT and different vertebrate lamin chromatin binding regions (66, 67). c, comparison of several A-T-rich DNA-binding proteins having sequence similarities to the A-T hook motif (69). A comprehensive survey of A-T hook proteins can be found in Ref. 70.
The hinge region is conserved in both inhibitory and noninhibitory serpins retaining the S → R capability (64) but is dramatically modified in serpins that lack both the inhibitory and the S → R functions (65).

Two prominent structural features distinguish MENT from other serpins. First, the MENT protein sequence is distinguished by a high content of positively charged amino acids and a high pI value (9.4) compared with a pI of 5–6.5 typical of serpins. As shown below, most of the basic MENT amino acids reside on the solvent-exposed surface of the protein and are probably involved in DNA binding. Second, the protein sequence between amino acids 61 and 91 (designated here as the M-loop) bears no resemblance to serpins (boxed portion in Fig. 4a). Amino acids 80–84 (asterisks in Fig. 4a) constitute a putative nuclear localization signal (NLS) typical of many nuclear proteins and identical to the NLS of *Xenopus* lamin II (66). The presence of an NLS is consistent with the nuclear localization of MENT (45, 46). The M-loop sequence has a significant similarity with a number of nuclear lamina proteins in the region including the NLS of *Xenopus* lamin II (67). The combination of the A/T hook motif with the nuclear lamin-like chromatin binding region in the M-loop domain may allow MENT to compete for nucleosomes with lamins and other proteins of the nuclear skeleton, thus providing a possible explanation for the previously reported activity of MENT toward dissociation of chromatin-nuclear matrix bonds in maturing erythrocytes (44).

The tertiary structures of a number of the serpin family members have been solved by x-ray crystallography (61, 62, 73) and show a high degree of structural conservatism. For example, the backbone of horse leukocyte elastase (74) can be superimposed almost precisely with that of α1-proteinase inhibitor and α1-anti-chymotrypsin, although it has only about 30% sequence identity with the former. Thus, the polypeptide backbone folding of MENT can be predicted with high confidence based on its similarity to ovalbumin (35% identity), horse leukocyte elastase (48% identity), and other serpins. We employed ProModII software (55, 56) to build a three-dimensional model of MENT based on the three-dimensional structure of ovalbumin solved by x-ray crystallography in its native uncleaved conformation with 1.95-Å resolution (75).

The inferred three-dimensional model of MENT showing the main secondary structural elements is presented in Fig. 5A. The protein is roughly 40 × 55 × 70 Å in size. The three-
dimensional structures of the two domains marked by the dashed boxes are uncertain. One is the sequence between amino acids 352 and 379, which corresponds to the reactive center domain (R-loop) and which undergoes abrupt conformational transitions in serpins (61, 62). The other is the nonserpin M-loop domain between amino acids 61 and 91, which is modeled here as a protruding highly basic bipartite loop that could be involved in interactions with DNA phosphates in a manner similar to the DNA-interacting “wings” of histone H5 and transcription factor hepatocyte nuclear factor 3 (76). It should be noted that the propensity of this region to form either α-helix or β-strand is approximately twice as low as to form a random coil as determined by the approach of Garnier et al. (77). Therefore, the M-domain has little potential to form a stable secondary structure and, conceivably, may extend as much as 50 Å.

Most of the positively charged amino acids are exposed on the surface of MENT and may interact with DNA phosphates through ionic bonds. A molecular surface potential model of MENT prepared using the GRASP program (57) shows a strong clustering of positive and negative charges on the protein surface. MENT appears to be a bipolar protein in which the basic amino acid clusters tend to localize closer to the M-loop domain, while the acidic and neutral clusters occupy the other side of the molecule close to the R-loop (Fig. 5B). The highly basic amino acid clusters are located on a convex protein surface, suggesting that this domain may be inserted between negatively charged molecules, perhaps between the nucleosome linkers, which are closely apposed at the entry/exit site of linker histone-containing nucleosomes (5). The negatively charged area in the vicinity of the R-loop may bind to basic

**Fig. 6.** MENT synthesis from cDNA transfected in COS-7 cells. Western blot of SDS-polyacrylamide gel electrophoresis of proteins isolated from chicken granulocyte chromatin (lane 1) and from the nuclei of COS-7 cells transfected with a control vector plasmid (lane 2) and plasmids containing MENT ORF without an active promoter (lane 3) and under the cytomegalovirus promoter (lane 4) (detection with anti-MENT antibodies).

**Fig. 7.** Immunolocalization of MENT ectopically expressed in COS-7 cells. Selected cells with relatively low (a–d) and high (e–h) levels of MENT immunostaining are shown. a, c, e, and g, cells stained with rabbit anti-MENT antibodies and fluorescein 5-isothiocyanate-conjugated goat anti-rabbit antibodies. d, cells stained with mouse anti-fibrillarin antibodies and donkey Texas Red-conjugated anti-mouse antibodies. b, f, and h, cells stained with Hoechst 33258.
regions of other nucleosomal proteins, such as histones or even MENT itself. It will be interesting to determine whether the R-loop of MENT, which is not charged (Fig. 5b) and thus does not form ionic bonds, is involved in the type of conformational transitions associated with this structural domain in the serpins.

Ectopic Expression of MENT cDNA—Actively proliferating cells of either avian or mammalian origin appear to be devoid of MENT. To explore the in vivo properties of MENT expression, we transfected a mammalian cell line, COS-7, with a MENT-expressing vector based on the cDNA sequence and containing its ORF placed downstream of a cytomegalovirus promoter. After transfection and incubation of the cells for 48 h, cytoplasmic and nuclear proteins were isolated and analyzed by Western blotting with anti-MENT antibodies. Nuclei (but not cytoplasm) of cells transfected with MENT cDNA produced a strong 42-kDa band (Fig. 6, lane 4) while the nontransfected cells, cells transfected with control vector DNA (lane 2), and cDNA without an active promoter (lane 3) were MENT-negative. This result shows that the cDNA encodes a unique protein with molecular weight, antigenicity, and nuclear localization fully consistent with the biochemical data and the sequence analysis of chicken MENT.

Many of the known heterochromatin-associated proteins are distributed nonrandomly within cell nuclei, often being associated with foci of condensed chromatin (28, 29). As is typical with the transient expression procedure, about 10% of total cells transfected with MENT-expressing vector developed various levels of MENT-specific signal (detected with anti-MENT antibodies but not with preimmune serum (45)). In all cells with a low and a moderate signal intensity, MENT was confined entirely within the nucleus. In nontransfected cells as well as in cells transfected with a control vector, no signal was detected.

Nuclei expressing relatively low amounts of MENT were similar in shape and size to typical COS-7 nuclei (Fig. 7, a–d). The strongest signal was associated with subnuclear organelles that also bind antibodies against fibrillarin, a nucleolar protein (e.g. see Ref. 52), showing that nucleoli are the preferential sites for initial MENT accumulation (Fig. 7d). Inside the nucleoli, Hoechst staining is relatively weak, indicating that MENT recognizes specifically the nucleolar material rather than merely following the local DNA concentration. It is noteworthy in this respect that the yeast SIR3 and SIR4 proteins involved in yeast “heterochromatin” are also found in the nucleolus, an association that has a powerful influence on the cell life span (78). A nucleolar localization of MENT has not been observed previously, since the terminally differentiated cells in which it has been studied (nucleated erythrocytes and granulocytes) lack nucleoli.

In nonnucleolar regions, MENT is distributed nonrandomly, forming foci of variable shape, many of which occur close to nucleoli. Staining by the DNA-specific dye Hoechst 33258 shows that the signals from Hoechst and from anti-MENT antibodies usually (but not always) coincide (Fig. 7, a, b, e, and f). Hoechst preferentially stains A/T-rich heterochromatin (79), and its co-localization with MENT foci may reflect a local heterochromatin recruitment by the protein.

Within the most extensively MENT-expressing cells (which also show MENT-positive staining in the cytoplasm), the nuclei are strongly fluorescent and consistently smaller in size than control cells. Also, more of the nuclear volume is occupied by MENT and Hoechst-positive foci (Fig. 7, e–h). Taken together with our previous results showing that an addition of 1–2 molecules of MENT/nucleosome can induce a significant chromatin condensation and nuclear shrinkage in vitro (46), this suggests that the reduction in nuclear size and the condensed nature of chromatin in these cells is the direct result of the high level of MENT and is likely to reflect its inherent chromatin-condensing properties.

**DISCUSSION**

Chromatin condensation in terminally differentiated cells is a widespread developmentally regulated phenomenon whose mechanism is poorly understood. Here we report the first study of isolated condensed chromatin where the highly compact state is linked to the accumulation of a single nonhistone protein, MENT. The evidence that MENT is both necessary and sufficient to effect chromatin condensation is compelling; its expression is strictly limited to the terminal stage of cell differentiation (44, 45); it is concentrated in peripheral heterochromatin (45, 46); it brings about chromatin condensation when either ectopically expressed in vivo (Fig. 7) or reconstituted with isolated nuclei in vitro (46); and it is the major nonhistone chromatin protein that is stably bound to compact polynucleosomes (Fig. 2). This rather simple experimental system allows a detailed study of the structure and formation of heterochromatin and also provides important general insights into the mechanisms of chromatin compaction and heterochromatin spreading.

Our cryoelectron microscopy observations allow a direct comparison between heterochromatin-derived granulocyte polynucleosomes and “euchromatin” from actively proliferating cells (Fig. 3). Two principal differences stand out: the heterochromatin sample shows a much closer packing of nucleosomes within the 30-nm fibers and also contains fibers that vary in diameter up to 50 nm. In previous work, changes in the compaction of polynucleosomes containing linker histones were shown to be effected by changes in the degree of electrostatic neutralization of DNA negative charges by counterions (14, 81). The increase in counterions is known to affect the entry-exit angle of the two linker DNA segments of a nucleosome consistent with a reduction in the mutual repulsion of the two linker DNA segments causing a longitudinal compaction (5, 18, 59). There is also an increased rate of self-association (14), apparently through lateral contacts between the fibers (14). In the present study, the different compaction levels of the heterochromatin and euchromatin samples can also be traced to differences in longitudinal and lateral compaction, but for granulocyte oligonucleosomes, high compaction levels do not require high salt but rather the presence of MENT, a basic protein in which the positively charged amino acids form potential DNA-interacting clusters at the surface of the molecule (Fig. 5B).

The presence in the heterochromatin-derived polynucleosomes of larger structures of varying diameter is indicative of inter- and intrafiber interactions, including “fold-back” regions and side-to-side self-associations, phenomena similar to those seen in tomographic reconstructions of whole starfish sperm nuclei (80). Self-association of chromatin fibers is also part of the continuum of salt-induced chromatin compaction (14, 15, 81) and likely to predominate in compact chromatin in vivo (82). Experiments in which we removed about 75% of MENT in order to release soluble chromatin from granulocyte nuclei, suggest that a more loosely bound fraction of MENT is responsible for the interfiber associations. We propose that both the lack of solubility of granulocyte chromatin with a full complement of MENT and the increased fiber diameter of MENT-enriched chromatin are due to the extensive interfiber interactions that occur in vivo within electron-dense masses of heterochromatin.

The MENT serpin homology suggests that in addition to DNA charge neutralization, the interfiber interactions may be dramatically strengthened by protein-protein interactions between the nucleosome-associated molecules of MENT. Indeed,
in some serpins, especially those with an impaired inhibitory function, the S → R transitions, instead of being intramolecular, involve spontaneous polymerization through an interaction of the R-loop domain of one protein with the A-sheet of another (83–85). In MENT, these domains are oriented away from the putative DNA-binding basic surfaces (Fig. 5F). Thus, the association of MENT with DNA would not interfere with potential protein-protein interactions. The conservation of the “hinge region” consensus at the basis of the R-loop is a very strong indication that the S → R capability may be present in MENT. Indeed, besides being conserved among the inhibitory serpins, this consensus is also present among noninhibitory serpins retaining the S → R capability, such as hormone-binding globulins (64), but is absent from ovalbumin and angiotensinogen, serpins that lack both the inhibitory and the S → R functions (65).

When isolated from nuclei, MENT behaves as a monomeric protein during gel filtration chromatography (data not shown). Thus, the suggested multimerization should be promoted by association of the protein with DNA and/or chromatin. Such an event has been recently observed in the ternary complex of MATa2 repressor with MCM1 transcriptional factor and DNA (86), where a “chameleon” transition between α-helical and β-strand conformations of a short amino acid motif in MATa2 has been revealed by x-ray crystallography. These authors pointed out that serpins are another group of proteins capable of “chameleon” transitions. It should be noted that although the change in secondary structure is an essential element of the serpin S → R transition, the latter also involves a larger scale rearrangement of the tertiary structure that may be important in chromatin remodeling.

Another property of MENT that may be indicative of its in vivo action is its ability to interfere in vitro with the partition of specific genes to the insoluble nuclear matrix fraction. Exogenously added MENT caused the c-myc “housekeeping” gene to move from the nuclear matrix fraction to the soluble fraction, but only after its expression had been shut down in the course of erythropoiesis, while the transcriptionally active H5 and β-globin genes showed no response to MENT (44). The presence in MENT of a nonserpin domain (M-loop) combining the features known to be involved in binding A’-rich DNA with localization in intranuclear foci stained with an A’-rich DNA-specific dye (Fig. 7) is consistent with a direct interaction of MENT with such DNA. In particular, MENT may recognize specific DNA sequences such as the c-Myc scaffold-associated region, which contains a prominent A’-rich DNA sequence (87). These elements may have a much stronger affinity for DNA-binding proteins in the context of a nucleosome array than as naked DNA as has been proposed for repeated A’-rich sequences (88), thus accounting for the apparent absence of sequence-specific interactions of MENT with DNA in vitro.2

In a recent paper (71) it has been shown that an artificial protein containing repeated A’-hook motifs, MATH20, can act as a general chromatin activator by interfering with position effect variegation in Drosophila. Based on their data, the authors suggested that the binding of hypothetical chromatin compacting proteins to scaffold-associated regions or certain A’-rich satellites could “lead to either chromatin folding, chromosome condensation (loopy), or formation of heterochromatin” (71). Their model, which involves a component with a striking similarity to the major properties of MENT, implies that the regulation of heterochromatin formation by competition between A’-binding activators and chromatin-condensing proteins may be a general phenomenon not restricted to terminal differentiation.

The remodeling of chromatin architecture associated with heterochromatin formation appears to be accomplished by a variety of proteins that act at different levels of chromatin organization. MENT, the first example that links heterochromatin with the higher order folding of chromatin fibers, is confined to terminally differentiated blood cells. Further work may reveal new proteins, related or unrelated to MENT, fulfilling similar functions in other cells and tissues. Their identification and study, using either biochemical and ultrastructural techniques similar to those that have proven successful here or genetic and in vivo approaches now facilitated by the cloning of the MENT cDNA, should provide a more general picture of the molecular interactions regulating development and position-specific chromatin condensation.

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