Metastable Macromolecular Complexes Containing High Mobility Group Nucleosome-binding Chromosomal Proteins in HeLa Nuclei*

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High mobility group nucleosome-binding (HMGN) proteins belong to a family of nuclear proteins that bind to nucleosomes and enhance transcription from chromatin templates by altering the structure of the chromatin fiber. The intranuclear organization of these proteins is dynamic and related to the metabolic state of the cell. Here we report that ~50% of the HMGN proteins are organized into macromolecular complexes in a fashion that is similar to that of other nuclear activities that modify the structure of the chromatin fiber. We identify several distinct HMGN-containing complexes that are relatively unstable and find that the inclusion of HMGN in the complexes varies according to the metabolic state of the cell. The nucleosome binding ability of HMGN in the complex is stronger than that of the free HMGN. We suggest that the inclusion of HMGN proteins into metastable multiprotein complexes serves to target the HMGN proteins to specific sites in chromatin and enhances their interaction with nucleosomes.

The nucleus of eukaryotic cells contains multiple activities that modify the structure of the chromatin fiber thereby affecting a variety of DNA-related activities such as transcription, replication, recombination, and DNA repair (1–5). Structural changes in the chromatin fiber are induced by enzymatic activities that target either the histones or the DNA in a variety of ways including the reversible post-translational modification of specific amino acid residues in histones by acetylation (6) and phosphorylation (7). Additional activities, some of which are ATP-dependent, remodel the structure of the nucleosome and facilitate access of regulatory factors to their cognate binding sites (8, 9). Biochemical and genetic studies indicate that most of these chromatin modifying activities are associated with other proteins in macromolecular complexes consisting of discrete subunits (1, 10–12). The proteins associated with the chromatin modifying activities may play a role in targeting them to specific modification sites. Interestingly, the protein subunits appear in several distinct complexes (1, 8, 13). It has been suggested that the sharing and mixing of subunits and their components among multiprotein complexes reflect the dynamic and modular nature of the macromolecular complexes containing the chromatin modifying activities. The dynamic aspect of these activities seems to play a role in the ability of a cell to respond to various metabolic demands (1, 2).

Changes in chromatin structure are also induced by structural proteins such as the high mobility group nucleosome-binding (HMGN) family (14–16), which are devoid of known enzymatic activities. HMGN proteins are a family of nuclear proteins found in the nuclei of all mammalian and many vertebrate cells (17). The nomenclature of the HMGN family has been changed recently (17 and the references therein). HMGN1 (former name HMG-14) and HMGN2 (former name HMG-17) proteins bind to the 147-bp nucleosome core particles, i.e. to the building block of the chromatin fiber, with no known specificity for the underlying DNA sequence (18). Distinct domains in the HMGN proteins interact with either the nucleosomal DNA or with the amino termini of the core histones, reduce the compaction of the higher order chromatin structure, and enhance transcription and replication from chromatin templates (19–22). Recent experiments using fluorescence loss in photobleaching and fluorescence recovery after photobleaching imaging techniques with specific HMGN point mutants provided direct evidence that in living cells HMGN proteins interact with chromatin through a specific nucleosome binding domain (23).

Central questions regarding the cellular function of the HMGN proteins are the mechanism whereby they are targeted to binding sites in chromatin and whether these binding sites are HMGN-specific. Photobleaching experiments demonstrated that in living cells these proteins move rapidly throughout the entire nucleus and therefore may reach their binding sites in a diffusion-driven manner, by random chance (24). A macromolecular HMGN complex was detected in HeLa nuclear extract, suggesting that, like other chromatin modifying activities, HMGN proteins may function in the context of macromolecular complexes (18). In this respect the mechanism of action of HMGN, and perhaps that of other structural chromatin-binding proteins, may be very similar to that of the histone acetyltransferases or the ATP-dependent chromatin remodeling activities.

In this report we demonstrate that a significant fraction of the nuclear HMGN is associated with other proteins into several metastable multiprotein complexes. The nucleosome binding ability of the HMGN in the complexes is higher than that of free HMGN. The low stability of the HMGN macromolecular complexes may enable the proteins to shuttle between complexes depending on specific cellular requirements. We suggest that HMGN proteins associate dynamically with multiple mac-

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‡ The abbreviations used are: HMGN, high mobility group nucleosome-binding; AEBSP, 4-[(2-aminoethyl)benzenesulfonyl] fluoride; EMSA, electrophoretic mobility shift assay; HA, hemagglutinin; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PBS, phosphate-buffered saline.
romolecular complexes and that these associations may affect the interaction of the proteins with their chromatin targets.

MATERIALS AND METHODS

Nuclear Extracts from HeLa Cells

HeLa S3 cells were grown in 5% fetal calf serum, minimal essential medium Joklik and harvested at a 5 x 10^6/ml density. Nuclear extracts were performed as described (25). Briefly, cells were washed with buffer A (10 mM HEPES-Na, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol with proteinase inhibitor mixture, Roche Molecular Biochemicals), suspended with Dounce homogenizer. The cells were disrupted with a tight type Dounce homogenizer, and nuclear proteins and their complexes were extracted with buffer C250 or C350 (final concentrations 20 mM HEPES-Na, pH 7.5, 25% glycerol, 1.5 mM MgCl₂, 250 or 350 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and a mixture of proteinase inhibitors).

A Size Exclusion Chromatography

FIG. 1. HMGN proteins in nuclear extracts are found in high molecular mass complexes. A, 0.25 M NaCl nuclear extracts from HeLa cells applied to Zorbax GF450 size exclusion columns. Shown is the OD280 profiles of the untreated (--), RNase-digested (—), or the DNase-digested (—) extracts. HMW and LMW denote, respectively, the presence of HMGN in high and low molecular mass fractions. B, Western slot blot analysis with anti-HMGN2, of selected fractions (indicated on top of the slots) from the Zorbax columns. Equal amounts of the extract have been fractionated, and the equal volume aliquots were immobilized. Fraction were from 1, untreated extracts; 2, DNase I-digested extracts; 3, RNase-digested nuclear extracts. Note that the nuclease digestions did not change the elution profile of the HMGN protein. C, formaldehyde cross-linking of the high and low molecular mass fractions. The high and low molecular mass fractions were dialyzed against amine-free buffer and treated with 0, 0.03, 0.1, and 0.3% formaldehyde (lanes 2–5 and 6–10, respectively). The reaction mixtures were fractionated on 10% SDS-polyacrylamide gels, and the presence of HMGN2 was visualized by Western analysis. Lanes 1 and lane 6 show Coomassie-stained proteins from the high and low molecular mass fractions prior to cross-linking. Note that both fractions contain multiple proteins. With increased cross-linking, in the high molecular fraction, but not in the low molecular mass fraction, the amount of free HMGN2 decreases, and a new, high molecular mass HMGN2-containing band appears (arrow, lanes 2–5). The HMGN2 protein in the low molecular mass fraction (lanes 7–10), although co-isolating with other proteins (lane 6), remains uncross-linked.

B Western with antiHMGN2 after crosslinking

C Western with antiHMGN2 after crosslinking

Formaldehyde Cross-linking of the HMGN1/N2 Complexes

The HMGN1-containing protein complexes and pure HMGN1 protein were incubated with 0.03, 0.1, and 0.3% paraformaldehyde in 20 mM HEPES, pH 7.8, 1 mM EDTA buffer for 30 min at room temperature. The products of the cross-linking were analyzed by Western blotting.

Two Schemes of Conventional Biochemical Purification of the HMGN1/N2 Complex

The chromatographic media used for the two purification schemes detailed in the text are indicated in Figs. 4 and 5. In each case, the preparations were dialyzed against the starting buffer of the next chromatographic step and then concentrated on spin dialysis columns. All buffers used for chromatography contained 0.1 mM phenylmethylsulfonyl fluoride. Fractions of interest were desalted or concentrated with CentriPrep or Centricon (10,000 cut-off) spin dialysis tubes. HMGN1 or HMGN2 in the various fractions was detected by Western
**Affinity Chromatography for the Isolation of the HMGN1 and N2 Complexes**

**Cell Culture**—HeLa cells were established to express stably HMGN1 or HMGN2 tagged at the carboxyl terminus with both FLAG and HA peptide tags by retroviral transduction (26). Cells were cultured in Dulbecco’s modified Eagle’s medium with 10% (v/v) fetal bovine serum (Invitrogen) and harvested after growing to confluence. The nuclear protein was extracted in HGEK 100 buffer (20 mM HEPES, pH 7.6, 10% glycerol, 100 mM KCl, 0.2% EDTA, 2 mM dithiothreitol, 0.1 mM AEBSF, 10% glycerol, at a flow rate of 0.5 ml/min.

**Affinity Purification of HMGN1 and N2 Complexes**—Most of procedures were carried out as recommended in the manufacturers’ protocols (Sigma and Roche). Anti-FLAG-agarose (Sigma) preequilibrated with HGEK 100 buffer was added to the nuclear extracts and incubated for 10 h at 4 °C with rotatory shaking. Agarose beads were recovered by centrifugation and washed twice with HGEK 100 buffer. To release the bound proteins the resins were incubated for 4 h with 150 μM FLAG peptide, and the resin was removed by centrifugation. The release of the protein was followed by Western blot with specific antibodies. The proteins released from FLAG-agarose were applied to anti-HA-agarose (Roche) and the resin treated as for the FLAG affinity procedure except that the proteins were eluted with 100 μM HA followed by 200 μl of 0.1 M glycine HCl, pH 3.5.

**Probing Complexes with NaCl, Urea, and Nucleases**

HeLa nuclear extracts, or specific fractions recovered from the size exclusion columns, were either concentrated or adjusted to the indicated NaCl or urea concentration and reloaded onto the same columns. The presence of HMGN in the various chromatographic fractions was detected by Western analysis after fractionation on 15% SDS-PAGE.

**Mass Spectroscopy for Peptide Fingerprinting**

The proteins from fractions of interest were resolved by electrophoresis in 15% SDS-PAGE, the gel stained with GelCode (Pierce), and bands of interest excised from the gel and sent to the PDTC facility at Rock-
Macromolecular Complexes Containing HMGN Proteins

fig. 3. Affinity chromatography purification of HMGN1/N2 multiprotein complexes. A, immunofluorescence analysis of transfected cells with anti-FLAG and anti-HMGN antibodies demonstrates that the tagged HMGN proteins enter the cell nucleus. B, Western analysis of nuclear extracts indicates that the tagged HMGN proteins are expressed at levels comparable with the untagged, endogenous HMGN proteins (note equal amounts of tagged and untagged endogenous protein in lane 2 and that the tagged proteins are purified efficiently by the affinity column). Lanes 1, 3, and 5 are extracts from mock transfected cell. Lanes 2, 4, and 6–9 are extracts from HMGN1-transfected cells. The antibodies used to develop the Western blots are indicated at the top of each blot. The affinity resin from which the material tested was eluted is indicated in lanes 5–8. Lane 9 is material not adsorbed on the FLAG affinity column. The total absence of tagged HMGN1 and the presence of endogenous untagged protein indicate efficient binding of the tagged protein to the affinity column. C, the protein composition of the eluates from the final affinity column (anti-HA) as revealed by Coomassie Blue staining after fractionation on a 5–20% gradient SDS-polyacrylamide gel. The extracts from which the proteins were purified are indicated at the top of each lane. The dots indicate two bands enriched in the cells transfected with the tagged HMGN proteins. These two bands were analyzed by MALDI-TOF. D, Western analysis demonstrates enrichment of hnRNPA1 and partial enrichment of annexin II in HMGN-containing complexes. Lanes 1–4 are extracts from mock transfected cells (lane 1), from cells expressing tagged HMGN1 (lane 2), from proteins recovered from the affinity columns after purification of mock transfected cells (lane 3), and from proteins recovered from the tagged HMGN1-expressing cells (lane 4). The antibodies used for each strip are indicated on the right.

Confocal Microscopy
HeLa cells grown on coverslips were washed with phosphate-buffered serum (PBS), fixed with 4% formaldehyde in PBS for 10 min at room temperature, washed with PBS, and incubated for 2 min in PBS containing 0.1% Triton X-100, 1% fetal bovine serum, 0.1% NaN3 (TNBS buffer). The anti-HMGN1, or anti-FLAG antibody (at about 0.1 µg/ml in TNBS), treatment was done overnight at room temperature in a moist chamber. Secondary antibody treatment was the same as primary treatment. After the final wash steps, coverslips were inverted onto glass slides using the ProLong anti-fade reagent (Molecular Probes) as the mounting medium. Fluorescent cells were examined with an epifluorescence microscope (Optiphot; Nikon) equipped with a confocal system (MRC-1024; Bio-Rad). Sequential excitation at 568, 488, and 647 nm was provided by a 15-w krypton-argon laser (American Laser, Salt Lake City, UT), and sequential images were collected using LaserSharp software (Bio-Rad).

Gel Mobility Shift Assay with Nucleosome Core Particles
Pure recombinant HMGN proteins or complexes containing HMGN proteins were incubated at low ionic strength, under noncooperative binding conditions (27), with either unlabeled or 32P end-labeled core particles, prepared from chicken blood as described previously (28). The total concentrations of the components of the reaction were 0.5 × TBE, chicken blood core particles (either 0.2 ng/µl 32P end-labeled or 100 ng/µl unlabeled), 1% Ficoll, and 1 µg/µl tRNA. The total reaction volume was 10 µl. The mobility shift assay was performed on a native 4% polyacrylamide gels in 0.5 × TBE. For Western analysis, the gels were transferred onto polyvinylidene difluoride membranes by the semidry transfer technique, using 5% methanol and 1 × Tris-glycine buffer, pH 8.3, and HMGN proteins were visualized with a mixture of affinity pure anti-HMGN1 and anti-HMGN2 antibodies.

RESULTS
HMGN Proteins Are Incorporated into Large Multiprotein Complexes—In the initial steps to determine whether HMGN1 and HMGN2 proteins are associated with other cellular components in macromolecular complexes, we fractionated HeLa S3 nuclear extracts on Zorbax GF450 or Superose 6 size exclusion columns. Western analysis of the chromatographic fractions with antibodies specific to either HMGN1 or HMGN2 indicated that most of the protein was found in two peaks (Fig. 1). The first peak eluted close to the void volume and contained high molecular mass complexes (HMW in Fig. 1A) that were larger than 500 kDa, whereas the second peak eluted near the salt volume, where low molecular mass components elute (LMW in Fig. 1A). The elution volume of the second peak was
the same as that of marker recombinant HMGN proteins (not shown), indicating that this peak contains free, uncomplexed HMGN proteins. Quantitative analysis of the HMGN proteins in the two main chromatographic fractions indicates that in nuclear extracts prepared by extraction with 0.35 M NaCl (see "Materials and Methods"), about 40% of the HMGN is in high molecular mass fractions. In nuclear extracts prepared by extraction with 0.25 M NaCl, ~60% of the HMGN proteins fractionate as a large molecular complex (not shown).

Because HMGN proteins are highly basic and because nuclear extracts may contain nucleic acids, we examined the possibility that these proteins are found in nucleoprotein complexes. To test this possibility, the nuclear extracts were digested with either DNase I or RNase, the digests were refractionated on the same exclusion columns, and the HMGN content of each chromatographic fraction was estimated by Western analysis. The optical density of the RNase-digested, but not that of the DNase I-digested, HeLa nuclear extract changed, indicating that indeed, the nuclear extracts contained RNA. However, the elution profile of the HMGN proteins was not affected by nuclease digestions (Fig. 1B), a clear indication that HMGN proteins in the HMG fraction are not associated with nucleic acids in a nucleoprotein complex.

To verify that in nuclear extracts HMGN proteins are present as macromolecular complexes and associated with other proteins, we treated both the high and low molecular mass fractions with various concentrations of formaldehyde, fractionated the reaction mixture by SDS-PAGE, and visualized the HMGN in the various fractions by immunoblotting. The high and low molecular mass fractions contained similar amounts of HMGN proteins (compare lanes 2 and 7 in Fig. 1C) and many additional proteins (lanes 1 and 6 in Fig. 1C) that potentially could cross-link with HMGN1. Coomassie Blue analysis of SDS-polyacrylamide gels on which the cross-linked reaction mixture was fractionated indicates that both the high and low molecular mass fractions produced multiple complexes. Western analysis of these gels clearly indicated that only the HMGN proteins in the high molecular mass fraction cross-linked into higher molecular mass bands. As illustrated with HMGN2, exposure of the HMGN-containing complex in the high molecular mass fraction (Fig. 1C) to increasing concentrations of formaldehyde led to a gradual decrease in the detectable amount of free HMGN and a concomitant increase in the amount of immunoreactive high molecular mass components (arrow, Fig. 1C). In contrast, formaldehyde cross-linking of similar amounts of HMGN2 in the low molecular mass fraction did not change the amount of free protein and did not produce high molecular mass immunoreactive components (Fig. 1C). Thus, the HMGN in the high molecular mass fraction is complexed with other proteins, and the short range cross-linker formaldehyde cross-links it to the protein partners, whereas the HMGN in the low molecular mass fraction is not in close contact with other proteins and does not cross-link into higher molecular mass complexes. Taken together, the results indicate that a significant portion of the nuclear HMGN proteins is found in large macromolecular complexes and that within these complexes the HMGN proteins make protein-protein contacts.

Characterization of the HMGN Macromolecular Complex—Fractionation of HeLa 0.25 M NaCl nuclear extracts on Superose 6 size exclusion columns also indicated that a large part of the nuclear HMGN proteins are found in high molecular mass
fractions. Traces of HMGN proteins were also detected in fractions of intermediate molecular mass which eluted between the void and salt volumes. Upon rechromatography on the same columns, the high molecular mass complexes dissociated, and most of the HMGN proteins were found in either the intermediate or the low molecular mass fractions (Fig. 2B). Unexpectedly, when the high molecular mass fraction was made 1 M in NaCl prior to rechromatography on the Superose 6 columns, the relative amount of HMGN protein found in the high molecular mass fraction increased, and only a little HMGN protein was detected in the low or intermediate molecular mass fractions (strip 2, Fig. 2C). The same effect was seen upon exposure of the high molecular mass to 2 M NaCl (strip 3, Fig. 2C), suggesting that the presence of HMGN proteins in a macromolecular complex is stabilized by hydrophobic interactions. Indeed, treatment of the nuclear extracts with either 4 or 8 M urea dissociated the high molecular mass complex, and most of the protein was found in low molecular mass fractions. We conclude therefore that the inclusion of HMGN protein in the high molecular mass complex is stabilized by hydrophobic rather than ionic interactions.

Multiple HMGN Protein Partners—To facilitate the isolation and characterization of HMGN-containing multiprotein complexes, we stably transfected HeLa cells with vectors expressing either the double tagged HMGN1-FLAG-HA or HMGN2-FLAG-HA protein. The tags were placed at the carboxyl terminus of the proteins so as not to interfere with their nuclear localization signal, which is located in the amino terminus (29). Immunofluorescence analysis revealed that the tagged proteins entered the nucleus (Fig. 3A). Western analysis of cell extracts indicates that the tagged HMGN proteins were expressed at high levels, comparable with the levels of the endogenous HMGN proteins (Fig. 3B, lanes 1, 2, 4, and 6). Nuclear extracts prepared from these cells were treated with affinity resins containing anti-FLAG antibodies, and the material eluted from these columns was reapplied to resins containing anti-HA antibodies. Western analysis with the affinity-purified materials indicated that indeed hnRNPA1, hnRNPA2/B, was significantly enriched in the affinity-bound proteins from cell extracts expressing the tagged HMGN proteins (Fig. 3D). Annexin II was also enriched in the HMGN-expressing cells but to a much smaller degree (1.6–2.3-fold more enrichment). Thus, in the nucleus, hnRNPA1 and perhaps annexin II may be associated with HMGN proteins.

Affinity chromatography was used previously to purify successfully multiprotein complexes containing activities that modify chromatin components (26, 33). In the case of HMGN proteins the number of bands copurifying through two sequential affinity purification steps with the double tagged proteins was very large. Two possible explanations could account for the large number of bands copurifying with the HMGN proteins. One possibility is that the proteins interact with other proteins and form a very large complex containing many components. Alternatively, the proteins are found in multiple complexes, each containing a characteristic set of proteins. To distinguish between these two possibilities and to characterize further the multiprotein complexes containing HMGN proteins we subjected the HeLa nuclear extracts to several purification schemes.

Multiple, Metastable HMGN-containing Protein Complexes—The various steps of one of these procedures used for HMGN2 is illustrated in the flow chart on the right. A 0.25 M NaCl nuclear extract was applied to a Mono S column eluted with a linear 0.1–1.0 M NaCl, pH 6, gradient (see “Materials and Methods”). Most of the HMGN2 was found in the fractions eluting after 4 and 32 min. Each of these two fractions was applied to a Superose 6 column. The fraction at 32 min yielded one major peak at position Su70, which corresponds to the position of free marker recombinant HMGN2. The fraction at 4

![HeLa nuclear extract](image-url)
min produced three major peaks, Su30, Su60, and Su70, which correspond to the high, intermediate, and low molecular mass fractions described in Fig. 2. Fractions obtained from Superose 6 columns were applied to a Mono Q column. On this column the Su30 fraction produced two major HMGN2-containing fractions named MQ24 and MQ29; the Su60 fraction produced fractions MQ10 and MQ24, and fraction Su70 produced fraction MQ24. Each of these MQ fractions was applied to a heparin-agarose column and produced several fractions, which were named according to their elution time (see Fig. 4). Analysis of the elution pattern on the heparin-agarose columns reveals that HMGN2 was detected in the fractions eluting at positions 49, 59, 72, and 82 (named He49, He59, He72, and He82, respectively). The fraction eluting at position 59 is free HMGN2 because recombinant marker HMGN2 elutes at the same position. Fractions He49, He72, and He82 contained at least 19, 9, and 17 major protein bands, respectively, as determined by silver staining of polyacrylamide gels (Fig. 4B). Additional faint staining bands were detectable, suggesting that these complexes were still not pure. Indeed, upon rechromatography these peaks produced additional complexes (not shown).

Analyses of the fractionation procedure in Fig. 4A suggest that the cell extract contains free HMGN2 and at least three distinct HMGN2-containing multiprotein complexes. These three multiprotein complexes were named according to their elution from the heparin-agarose columns as He49, He72, and He82 (symbolized as a diamond, square, and circle, respectively). The fractionation scheme of the complexes is indexed by the symbols in Fig. 4A. It is important to note that all of the complexes contain HMGN2, as determined by Western analysis. We also note that most complexes, when applied to the various columns, yielded free, uncomplexed HMGN2. Most probably the free HMGN2 results from the dissociation of the complex during chromatography, another indication that the association of the protein in the complex is not very stable. We also note that the majority of the bands were unique to a single complex. The fact that each complex contained a unique set of proteins argues strongly that the complexes are distinct and not derived from each other.

A different fractionation scheme provided further support that HMGN proteins are found in multiple complexes. In the fractionation scheme depicted in Fig. 5 the HeLa extract was first applied to CM-Sepharose column and eluted with a stepwise 0.1–1 M KCl gradient, pH 7.5. Each of the fractions containing HMGN1 protein was then applied to Mono Q and eluted with a 0.1–1 M linear NaCl gradient, pH 8.8. Based on the results obtained from this fractionation procedure we conclude that the HeLa cell extracts contain free HMGN1 and at least five HMGN1-containing multiprotein complexes. These complexes are named according to their elution from the Mono Q columns as 27 (diamond), 31 (circle), 35 (pentagon), 39 (square), and 55 (oat). Polyacrylamide gel analysis of these fractions revealed that each contained multiple proteins, most of which were unique to each fraction (not shown). The results of this fractionation scheme are in full agreement with the fractionation scheme described above for HMGN2.

The complexes identified in Figs. 4 and 5 are the main, but not the only, HMGN-containing complexes because throughout the fractionation procedure HMGN was detected, albeit in lower relative amounts, in additional fractions not illustrated in the figures. Taken together, the data indicate that in HeLa nuclei the HMGN proteins are found in multiple, relatively metastable, multiprotein complexes.

**Redistribution of HMGN Proteins between Free and Complexed Forms after Treating the Cells with α-Amanitin**—The complexes...
Redistribution of the HMGN proteins between free and complexed forms after treating the cells with \(\alpha\)-amanitin

HeLa S3 cells were cultured in medium containing 20 \(\mu\)g/ml \(\alpha\)-amanitin (Sigma) for 4 h (34). Nuclear extracts prepared from these and from control cells grown without \(\alpha\)-amanitin were fractionated on Mono S HR5/5 column. On this column, the retention time of the major HMGN complexes is 4 min, and that of free HMGN is 32 min (see Fig. 4). The amount of HMGN in each fraction was quantified by immunoblotting.

| HMGN1 | HMGN2 |
|-------|-------|
| No treatment | \(\alpha\)-Amanitin | No treatment | \(\alpha\)-Amanitin |
| % | % | % | % |
| Complexed HMG | 68 | 47 | 45 | 33 |
| Free HMG | 32 | 53 | 55 | 67 |

These findings raise the possibility that the metabolic state of the cell affects the interaction of HMGN with various macromolecular complexes. To test this possibility we prepared nuclear extracts from HeLa cell grown in either the presence or absence of 20 \(\mu\)g/ml \(\alpha\)-amanitin and fractionated the extracts on Mono S columns. In these columns the retention time of HMGN bound to macromolecular complexes is 4 min, whereas that of free HMGN is \(\sim\)32 min. Quantitative immunoblotting of the relative amounts of HMGN in these two fractions indicates that transcriptional inhibition led to a significant increase in the relative amount of “free” HMGN (Table I). For HMGN1, the ratio of complex-bound to free protein changed from 2 (68:32, see Table I) in the control cells, to 0.9 (47:53) in the \(\alpha\)-amanitin-treated cells. For HMGN2, the ratio changed from 0.8 to 0.5. We conclude therefore that the metabolic state of the cell may affect the association of the proteins with various complexes.

Enhanced Nucleosome Core Particle Binding by HMGN in a Multiprotein Complex—HMGN proteins are chromatin-binding proteins that specifically recognize the 147-bp nucleosome core particle (35, 36). Our finding that a large fraction of the HMGN proteins is organized into macromolecular complexes raises the possibility that the HMGN proteins reach their chromatin targets in association with other proteins rather than as free, uncomplexed proteins. We used mobility shift assays to compare the nucleosome core binding ability of free HMGN protein with that of the HMGN proteins in a macromolecular complex. \(^{32}\)P end-labeled nucleosome cores were complexed under noncooperative binding conditions (27) with increasing amounts of pure HMGN proteins to give the characteristic shifts indicative of the binding of either one or two HMGN molecules to the core particle (Fig. 6A, left). In parallel, appropriate amounts of the HMGN-containing complex He72 (see Fig. 4) were added to the same batch of end-labeled nucleosome cores to give comparable mobility shifts (Fig. 6A, right). The relative amounts of HMGN proteins in the He72 complex were estimated by Western analysis with the same volume aliquot as used for the mobility shift assays. As indicated by the results in Fig. 6A, the signals received from the HMGN in the complex were significantly lower than that obtained with the free, purified HMGN protein. Although Western analysis with enhanced chemiluminescent reagents does not accurately reflect the absolute amount of antigen, it is clear that the amount of HMGN in the complex required to produce the mobility shift was lower than that of the free HMGN (Fig. 6A). These results suggest that the HMGN in the complex binds to nucleosome cores with a higher efficiency than the free HMGN proteins.

To examine this possibility further we compared the nucleosome core binding ability of equal amounts of free and complexed HMGN proteins (Fig. 6B). In these experiments the amount of He72 complex used was calibrated to give Western signals comparable with that of free HMGN. Note that at low amount of HMGN protein, the signals were still in the relatively linear range (compare the Western blots in lanes 2 and 3 with those in lanes 2’ and 3’). Thus, the nucleosome cores were reacted with the same amount of HMGN protein, either free or in a complex. To estimate directly only the HMGN molecules that are actually bound to the core particles, the complexes were transferred electrophoretically from the gels to a membrane, and the amount of HMGN associated with the shifted particles was visualized by immunoblotting. The HMGN signal obtained from the nucleosome cores shifted with the complex was significantly stronger than that obtained from the nucleosomes shifted with the free HMGN protein (compare lanes 2–5 with 2’–5’ in the EMSA/Western, Fig. 6B). Free HMGN produced mostly complexes containing one HMGN molecule/nucleosome core particle, whereas similar amounts of HMGN in the complex produced mobility shifts indicative of two molecules of HMGN/nucleosome core particle. These results provide direct evidence that the HMGN associated with other components in a macromolecular complex binds to nucleosome core particles more efficiently than the free, uncomplexed HMGN protein. Based on previous calculations (27) and assuming that the Western analysis accurately reflects the amount of protein, we estimate that the affinity constant for nucleosome binding of the HMGN in the complex is an order of magnitude higher than that of the free HMGN.

DISCUSSION

This report describes the first systematic study of multiprotein complexes containing HMGN chromosomal proteins. We find that in HeLa nuclei a large fraction of the HMGN chromosomal proteins is found in multiple, metastable macromolecular complexes. The nucleosome binding ability of the HMGN in the complex is enhanced, compared with free, purified HMGN proteins. These findings provide new insights into the intranuclear organization and target interactions of the HMGN and perhaps other chromatin-binding structural proteins.

Multiple HMGN Complexes—Three main types of experimental evidence support the conclusion that a significant fraction of the HMGN proteins is associated with other proteins in a macromolecular complex. First, fractionation of extracts prepared from HeLa nuclei with 0.25–0.35 \(\mu\)M NaCl concentrations, on several types of size exclusion columns, invariably demonstrated that a large portion of the HMGN proteins is found in high molecular mass fractions. Second, nuclease digestions and exposure to very high ionic strengths or urea solutions indicate that in these complexes the HMGN proteins are not associated with nucleic acids. Thus, the presence of HMGNs in the high molecular mass fractions is not the result of nonspecific association with nucleic acids or with chromatin fragments that could inadvertently be present in the nuclear extracts. Third, formaldehyde treatment of HMGN in the high, but not low, molecular mass protein fraction from the size exclusion columns cross-links the HMGN protein into large complexes. Thus, in the high molecular mass fraction, the HMGN proteins are in close contact with other nuclear proteins.

The various purification schemes (Figs. 3–5) provide further evidence that HMGN is in association with many types of proteins. Sequential affinity chromatography with FLAG- and HA-tagged proteins was used previously to identify and characterize several multiprotein complexes containing chromatin modifying activities (26, 33). In most of these cases, a very large amount of nuclear extracts was used to purify relatively small quantities of a particular complex. In the case of the HMGN proteins, which may be more abundant than histone acetyltransferases or ATP-dependent nucleosome remodeling com-
plexes, the data suggest that relatively large numbers of protein are associated with HMGN in a form that is sufficiently stable to be recovered after two affinity purification steps. The number of polypeptides specific to or highly enriched in the affinity-purified complexes was too large to represent a single complex. More probably, HMGN are found in several complexes, a suggestion that is supported by the fractionation schemes presented in Figs. 4 and 5. Thus, although the enzymatic or ATP-dependent chromatin modifying activities may be found in one or a few major complexes, HMGN proteins are found in many complexes, none of which is predominant.

How many HMGN-containing macromolecular complexes are present in HeLa nuclei? We have identified at least five HMGN1 and three HMGN2-containing complexes. Some of these contain both of the HMGN proteins. Further fractionation and altered fractionation schemes suggested the presence of additional complexes. Most likely, the complexes that we identified are the most stable ones under the specific fractionation procedures used; however, they readily dissociate and release free HMGN proteins. The complexes are not derived from each other because each complex contains a unique set of polypeptides.

Origin of HMGN Complexes—Photobleaching experiments demonstrate that many nuclear proteins are highly mobile (24, 37). The HMGN proteins move throughout the nucleus in a random type motion, with an apparent diffusion constant of 0.5 μm²/s (24). Assuming a nucleus with a diameter of 15 μm and an abundance of ~10⁵ molecules of HMGN, it can be calculated (38, 39) that an HMGN molecule would collide with another protein of 10⁴ abundance every 2 s and with a protein of 10⁶ abundance every 0.2 s. These frequent collisions may lead to the generation of metastable complexes in a fashion similar to the formation of self-organizing nuclear structures (40). In this scenario, the HMGN proteins would associate transiently with specific proteins partners and exchange continuously among various multiprotein complexes. The complexes isolated would depend on the abundance of the protein partners, reflect the cellular requirement for interactions between HMGN proteins and other nuclear components and Drs. Fred Friedman and Yaffa Rubinstein for a critical review of the manuscript.

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