High Mobility Group 1 Protein Is Not Stably Associated with the Chromosomes of Somatic Cells

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Abstract. High mobility group 1 (HMG1) protein is an abundant and conserved component of vertebrate nuclei and has been proposed to play a structural role in chromatin organization, possibly similar to that of histone H1. However, a high abundance of HMG1 had also been reported in the cytoplasm and on the surface of mammalian cells. We conclusively show that HMG1 is a nuclear protein, since several different anti-HMG1 antibodies stain the nucleoplasm of cultured cells, and epitope-tagged HMG1 is localized in the nucleus only. The protein is excluded from nucleoli and is not associated to specific nuclear structures but rather appears to be uniformly distributed. HMG1 can bind in vitro to reconstituted core nucleosomes but is not stably associated to chromatin in live cells. At metaphase, HMG1 is detached from condensed chromosomes, contrary to histone H1. During interphase, HMG1 readily diffuses out of nuclei after permeabilization of the nuclear membranes with detergents, whereas histone H1 remains associated to chromatin. These properties exclude a shared function for HMG1 and H1 in differentiated cells, in spite of their similar biochemical properties. HMG1 may be stably associated only to a very minor population of nucleosomes or may interact transiently with nucleosomes during dynamic processes of chromatin remodeling.

HIGH mobility group 1 protein (HMG1)[1] is a very abundant and highly conserved component of chromatin which is present in all mammalian tissues and cells. Moreover, HMG1-like proteins also exist in yeast, protozoa, and plants (for reviews see Bustin et al., 1990 and Bianchi, 1995).

HMG1 contains two DNA-binding domains of the HMG box class: they bind with low affinity to single-stranded, linear duplex and supercoiled DNA (Sheflin and Spaulding, 1989; Stros et al., 1994) and with high affinity and specificity to DNA containing sharp bends or kinks, such as four-way junctions or DNA covalently modified with the antitumor drug cisplatin (Bianchi et al., 1989, 1992; Pil and Lippard, 1992). More generally, HMG1 has the ability to transiently introduce bends or kinks into linear DNA and therefore is functionally (but not structurally) similar to the prokaryotic proteins HU and IHF, which it can substitute in several in vitro reactions (for review see Bianchi, 1994).

The evolutionary conservation of HMG1 suggests that it serves an indispensable function. Roles have been suggested in DNA replication, chromatin assembly and disassembly (Bonne-Andrea et al., 1984; Waga et al., 1990; Travers et al., 1994), and transcription (Tremethick and Molloy, 1988; Singh and Dixon, 1990; Ge and Roeder, 1994; Stelzer et al., 1994; Shykind et al., 1995); however, none of these hypotheses has been confirmed unequivocally. More recently it has been proposed that HMG1 plays a role similar to that of histone H1 in the organization and/or maintenance of chromatin. Both HMG1 and histone H1 bind to bent DNA structures (Bianchi et al., 1989; Varga-Weisz et al., 1993), and both appear to interact with linker DNA sequences (Schröter and Bode, 1982; van Holde, 1988). Moreover, *Xenopus* HMG1 binds to nucleosomes in vitro in much the same way as histone H1 and appears to replace histone H1 during early embryogenesis (Dimitrov et al., 1993, 1994; Nightingale et al., 1996). Likewise, HMG-D, a *Drosophila* homolog of HMG1, associates with condensed chromatin during embryonal development and is gradually replaced by histone H1 after the midblastula transition (Ner and Travers, 1994).

The present study focuses on the subcellular localization of mammalian HMG1 and its association with chromo-

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1. *Abbreviations used in this paper*: HMG1, high mobility group 1 protein; LDH, lactate dehydrogenase.

somes and chromatin during interphase and metaphase. We show with different antibodies that in nondividing fibroblasts HMG1 is localized exclusively within the nucleus. During metaphase, HMG1, like many transcription factors, detaches from condensed chromosomes and diffuses to the cytoplasm. Histone H1, on the other hand, remains bound to mitotic chromosomes. Moreover, HMG1 is released from interphase nuclei if the membranes are permeabilized with detergents. Thus, the association of mammalian HMG1 with chromatin is much less stable than that of linker histone H1. We suggest that histone H1 prevents HMG1 from binding to nucleosomes and that HMG1 can have a role as a bulk structural protein of chromatin only when histone H1 is absent.

Materials and Methods

Preparation of Antibodies against HMG1

HMG1/M1-K89 and HMG1/M1-F147 were expressed and purified in *Escherichia coli* as previously described (Falciola et al., 1994). Antibody mAP-bA was raised by injecting BALB/c mice four times with 200 μg of HMG1/M1-F89 at 2 wk intervals. Chicken antibodies were raised by injecting 200 μg of HMG1/M1-F147 three times at 2 wk intervals. Antibodies mAP-bA and chIP-AB were affinity purified on the cognate native antigen immobilized on CL-4B Sepharose (Pharmacia Biotech, Piscataway, NJ) at the concentration of 1 mg/ml and eluted with 0.1 M glycine-HCl (pH 2.5). Antibody chWB-AB was affinity purified using a strip of Immobilon filter (Millipore Corp., Bedford, MA) bearing 2 mg of HMG1/M1-F147 transferred from an SDS-PAGE gel.

Other Antibodies

The mouse monoclonal IgM HBC-7, which specifically recognizes the NH_2-terminal region of histone H2B (Whitfield et al., 1986), was a gift from B.M. Turner (University of Birmingham, UK). The chicken anti-recAtn antibody was kindly provided by H. Rauvala (Biotekniiken Instituutti, Helsinki, Finland), the rabbit anti–rat H1 antibody by M. Bustin (National Cancer Institute, Bethesda, MD), and the rabbit anti-HMGI(Y) antiserum by D. Thanos (Columbia University, New York, NY). The biotinylated anti-HA mouse mAb 12CA5, recognizing the nonapeptide YPYDVPDYA, was purchased from Boehringer Mannheim Corp. (Mannheim, Germany).

Dot Blots, Immunoprecipitations, and Western Blots

For dot blots, purified proteins were spotted onto wet Immobilon filters (Millipore Corp.). For Western blots, samples were applied to 10% tricine–SDS–polyacrylamide gels (Schägger and von Jagow, 1987) and then electroblotted onto Immobilon filters. Filters were blocked by incubation for 1 h in TBST (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Triton X-100) containing 10% skim milk and probed with antibodies diluted in TBST containing 0.1% BSA.

For immunoprecipitations, 400 ng of purified proteins dissolved in 200 μl of IP buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, 1% BSA, 1 mM DTT, and 0.2 mM PMSF) were mixed with 25 μl chIP-AB and 20 μl (packed volume) of swollen protein A-Sepharose (Pharmacia Biotech) presaturated with rabbit anti–chicken Ig (Zymed Labs, Inc., S. San Francisco, CA). The suspension was incubated for 1 h at room temperature with mild agitation. The beads were centrifuged at 800 rpm in a refrigerated Eppendorf microfuge and washed three times with ice-cold IP buffer. The bound protein was eluted using 50 μl of SDS-PAGE loading buffer and analyzed by Western blotting.

For detection of chWB-AB, we used a rabbit anti–chicken IgY secondary antibody conjugated to alkaline phosphatase (Chemicon Intl., Inc., Temecula, CA), which was revealed with either BCIP/NBT for color reactions or CDP-Star (Boehringer Mannheim Corp.) for chemiluminescence. The mouse 12CA5 monoclonal antibody was detected by donkey anti–mouse Ig secondary antibody conjugated to HRP (Amersham Corp., Arlington Heights, IL). For histone H1 and HMG-I(Y) detection, incubation with the cognate rabbit primary antibodies was followed by incubation with donkey anti–rabbit Ig conjugated with HRP (Amersham Corp.). For lactate dehydrogenase (LDH) detection, the goat primary antibody was followed by incubation with rabbit anti–goat Ig conjugated with HRP (Southern Biotechnology, Birmingham, AL). HRP was revealed using a Western blotting system (ECL; Amersham Corp.).

Immunofluorescence

All cell lines were cultured as monolayers on glass coverslips. Cells were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. After rinsing, the coverslips were stored in PBS containing 0.02% sodium azide, no longer than 1 wk at 4°C before use. Different permeabilizing agents were tested: 0.1% NP-40, 0.1% SDS, 0.1% Triton X-100 (all for 5 min at room temperature) and methanol for 5 min at −20°C. Results were always comparable. The coverslips were then incubated with blocking solution (PBS containing 1% BSA and 0.05% Tween-20) followed by primary and secondary antibodies (FITC-conjugated goat anti–chicken Ig antibodies; Southern Biotechnology; and FITC- or rhodamine-conjugated goat anti–mouse Ig antibodies; Sigma Chemical Co., St. Louis, MO) and finally mounted on glass slides with PBS containing 25% glycerol, 100 mg/ml DABCO as antifading reagent, 0.02% sodium azide, and 100 μg/ml Hoechst 33258. Every incubation was followed by three rinses in PBS containing 0.05% Tween-20. Specimens were examined using an Olympus BH-2 fluorescence microscope with the standard filters for FITC, rhodamine, and Hoechst 33258 emission. Photomicrographs were taken with Ektachrome 400 HC films (Kodak) and either printed directly or scanned and processed using Adobe Photoshop software. Confocal laser scanning microscopy was performed with a Zeiss laser fluorescence microscope equipped with argon and helium lasers. Double fluorescence signals were separated by a two-band filter. The emitted signal was digitalized by Kallman filter collection, and each section was scanned eight times.

Plasmids

Plasmid p-mHMG1 consists of a 9-kb EcoRI–NsiI fragment containing the entire mouse *Hmg1* gene, cloned in pBlueScript. To obtain plasmid pmHMG1tag, the sequence 5′-TACCCATACGACGTCCCAGACTACGCT-3′, coding for the nonapeptide YPYDVPDYA (HA epitope), was inserted by mutagenic PCR into exon 2 of the *Hmg1* gene, between amino acids 1 and 2. This insertion was the only modification to the genomic fragment. The construct was completely sequenced to verify its identity. All oligonucleotides were purchased from Genset (Paris, France). DNA modification and restriction enzymes were from Boehringer Mannheim, Promega Biotech (Madison, WI), and New England Biolabs (Beverly, MA).

Nucleosome Electrophoresis Mobility Shift Assays

A 5′ labeled fragment, encompassing mouse rDNA sequences from −160 to +16, was generated by PCR and used for nucleosome assembly by salt dialysis. 5 femtomoles of rDNA was incubated with 200 ng of carrier DNA (phage λ DNA cut with HaeIII) and 100 ng of purified chicken histones in a final volume of 20 μl in a buffer containing 2 M NaCl, 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.1% NP-40, 1 mM β-mercaptoethanol. The preparations were dialyzed overnight against the same buffer containing 50 mM NaCl. Nucleosomes were then incubated with 0–500 ng of HMG1 for 15 min at room temperature. The samples were analyzed by electrophoresis on 0.7% agarose gels in 0.5× TBE. Gels were dried on paper and autoradiographed.

Cell Culture and Transfection

All cell lines were grown in DME supplemented with 10% newborn calf serum (NCF; GIBCO BRL, Gaithersburg, MD). Transfections were performed by the calcium phosphate method. NIH 3T3 clones stably transformed with pmHMG1tag were obtained by transfecting 5×10^5 cells with 20 μg of pmHMG1tag plasmid and 1 μg of pRSV-neo plasmid, followed by selection with 900 μg/ml G418 for 11 d.

Sucrose Gradient Fractionation of Chromatin

About 50 million NIH 3T3 fibroblasts grown to near confluence were washed with ice-cold RSB buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$) and resuspended in 25 ml of RSB buffer. The cell suspension was then supplemented with 5 ml of RSB buffer containing 1.2 mM PMSF and 6 μg each of leupeptin, pepstatin, and antipain, homogenized on ice by 20 strokes in a glass teflon homogenizer, and centrifuged at 500 *g*

for 5 min at 4°C. Nuclei were resuspended to a final absorbance of 3.8 at 260 nm in buffer M (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $CaCl_2$, 0.1% NP-40, and 6 μg each of leupeptin, pepstatin, and antipain) and digested in 400 μl aliquots for 5 min at 37°C with varying amounts of micrococcal nuclease (MNase; Worthington Biochemical Corp., Freehold, NJ). The results reported were obtained with 40 U/ml of MNase; different extents of digestion gave comparable results (not shown). The digestion was stopped and nuclei were lysed by the addition of 1.6 ml of 1.5 mM EDTA. The suspension was layered onto a 5–28% linear sucrose gradient (30 ml) prepared in 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, and protease inhibitors as above. The gradients were centrifuged at 24,000 rpm for 30 h at 4°C in a SW27 Beckman rotor. Fractions (1 ml) were analyzed for DNA content after phenol–chloroform extraction and for protein content after precipitation in 20% TCA.

Differential Permeabilization of Cells

NIH 3T3 fibroblasts were grown to subconfluence in 24-well plates (Falcon Plastics, Cockeysville, MD). Adherent cells were washed three times with ice-cold transport buffer (TB buffer), containing 20 mM Hepes, pH 7.3, 110 mM K-acetate, 5 mM Na-acetate, 2 mM Mg-acetate, 1 mM EGTA, 2 mM DTT, and pepstatin, antipain, and leupeptin at 1 μg/ml each (Adam et al., 1990). They were then incubated for 5 min on ice in 80 μl of TB buffer or TB buffer with 0.1% NP-40 or 40 μg/ml digitonin (Sigma Chemical Co.). Supernatants were recovered, and cell remnants were incubated for 10 min at 37°C with 80 μl of TB buffer plus 0.1% NP-40, 10 mM $MnCl_2$, and 20 μg/ml DNaseI (Boehringer Mannheim Corp.). SDS-PAGE loading buffer was then added to supernatants and cell remnants, and samples were analyzed by Western blotting.

Mitotic and interphase cells were obtained and processed essentially as described (Martinez-Balbás et al., 1995). NIH 3T3 fibroblasts, grown in 75-cm^2 flasks (Costar, Cambridge, MA) to ~70% confluence, were exposed to 50 ng/ml nocodazole (Janssen Chimica, Beerse, Belgium) for 16 h. Cells blocked in mitosis were detached by manual shaking of the culture flasks and recovered by centrifugation at 800 *g*. Interphase cells remained attached to the flask bottom and were recovered by mild trypsinization and centrifugation. Both cell populations were washed once in PBS, once in ice-cold TB buffer, and finally resuspended in 0.5 ml of ice-cold TB buffer. Small aliquots were stained with Hoechst 33258 and observed by fluorescence microscopy: >95% of the cells detached by shaking were found to have condensed chromosomes, against <2% of the cells that resisted detachment. Mitotic and interphase cells were permeabilized by adding one volume of ice-cold TB buffer containing 0.2% NP-40, 2 μg/ml each of leupeptin, pepstatin, and antipain, and 0.4 mM PMSF. Cells were then centrifuged for 3 min at 13,000 rpm in a refrigerated Eppendorf microfuge (Brinkman Instruments, Westbury, NY). Supernatants were recovered, and the original volume was reconstituted with TB buffer containing 0.1% NP-40, 10 mM $MnCl_2$, and 20 μg/ml DNaseI. The suspensions were incubated for 10 min at 37°C. SDS-PAGE loading buffer was then added to supernatants and cell remnants, and samples were analyzed by Western blotting.

Results

Anti-HMG1 Antibodies Stain the Cell Nucleus

Several previous cell fractionation and immunofluorescence studies indicated that HMG1 is located both in the cytoplasm and in the nucleus of mammalian cells (Bustin and Neihart, 1979; Isackson et al., 1980; Einck et al., 1984; Kuehl et al., 1984; Mosevitsky et al., 1989). Moreover, HMG1 has been localized to the surface of neural and fibroblastoid cells (Parkkinen et al., 1993). These results are not necessarily in conflict with HMG1's proposed role(s) as a DNA-binding protein but warranted a reconsideration of HMG1's subcellular localization.

Because of the high evolutionary conservation of HMG1, antibodies are hard to obtain, and even repeated injection

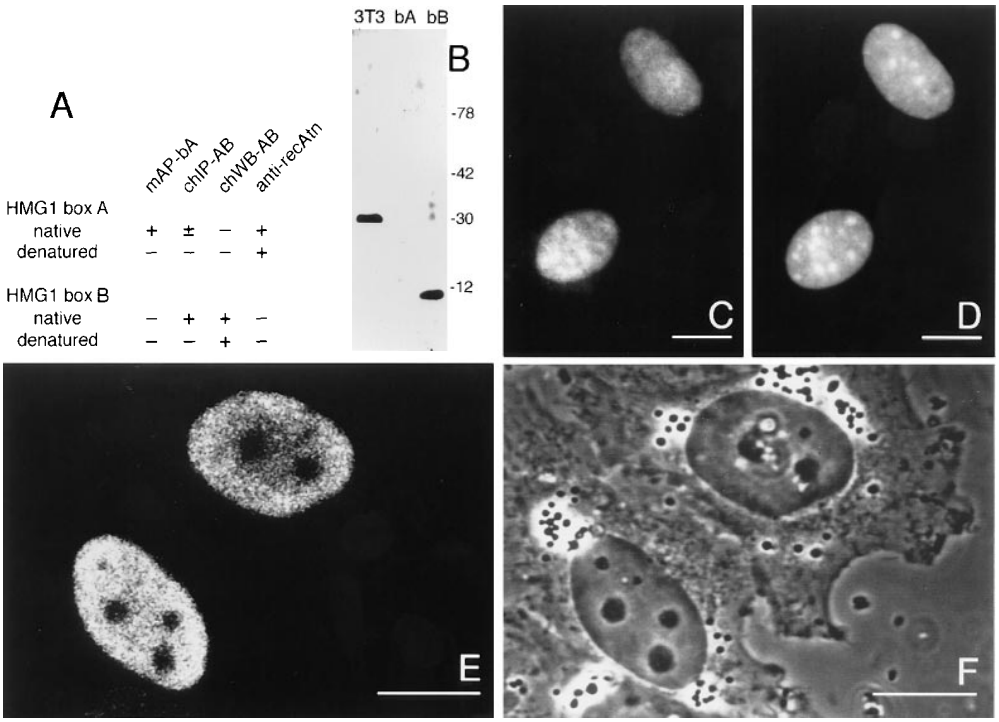

Figure 1. (*A*) Reactivity of the various anti-HMG1 antibodies used in this study. For each antibody preparation the species of origin is indicated (*m*, mouse; *ch*, chicken), and the ability (+) or inability (−) to recognize boxes *A* and *B* of HMG1 by Western blotting (*denatured*) or by immunoprecipitation (*native*) is also shown. The notation ± indicates that recovery of the HMG1bA polypeptide by immunoprecipitation is <5% under the conditions indicated under Materials and Methods. (*B*) Reactivity of the chWB-AB antibody in Western blots. Whole NIH 3T3 cells were lysed by addition of SDS-PAGE loading buffer, and 10 μg of total protein was loaded on a 10% tricine–SDS–polyacrylamide gel (lane *3T3*), alongside 20 ng of purified recombinant HMG1bA polypeptide (lane *bA*) or 20 ng of purified recombinant HMG1bB polypeptide (lane *bB*). (*C* and *D*) Anti-HMG1 antibodies stain the cell nucleus. NIH 3T3 cells were grown on glass coverslips, fixed with paraformaldehyde, permeabilized with 0.1% NP-40, and stained with anti-recAtn (*C*) and Hoechst 33258 (*D*). (*E* and *F*) Localization of HMG1 by confocal microscopy. HeLa cells were fixed with paraformaldehyde, permeabilized with 0.1% SDS, stained with mAP-bA antibody, and viewed in green fluorescence (*E*) or by phase contrast microscopy (*F*). Bars, 10 μm.

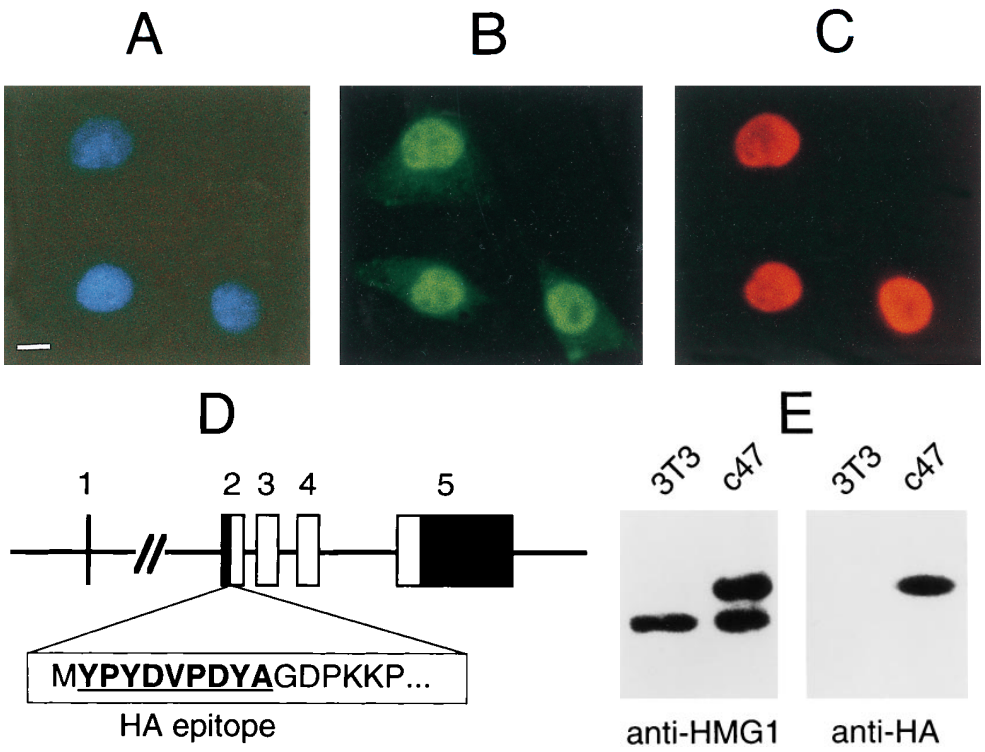

Figure 2. The product of the *Hmg1* gene localizes to the nucleus. NIH 3T3 fibroblasts stably transfected with the pHMG1tag plasmid were fixed and stained simultaneously with (*A*) Hoechst 33258, (*B*) anti-HMG1 antibody chIP-AB, and (*C*) monoclonal antibody 12CA5 recognizing the HA epitope. (*D*) Structure of the *Hmg1-tag* gene. The mouse gene *Hmg1*, which codes for protein HMG1, is transcribed under the control of its own strong promoter/enhancer. Exons are numbered. Black boxes represent untranslated sequences and white boxes translated sequences. Plasmid pmHMG1 was modified by the insertion of 27 bp coding for the HA epitope (*bold* and *underlined*) immediately after the ATG codon for the first methionine of HMG1 and in frame with the rest of the protein. A stable clone (*c47*) expressing HMG1tag approximately to the same level of unmodified HMG1 was selected. (*E*) Western blotting of whole cell extracts from wild-type fibroblasts (lanes *3T3*) and from the stable transfected cells (lanes *c47*). The anti-HMG1 antibody chWB-AB (*left*) recognizes in the same way HMG1 and HMG1tag; the monoclonal antibody 12CA5 recognizes the HA epitope (*right*). Protein HMG1tag runs slightly slower than wild-type HMG1 in tricine–SDS-PAGE because of the addition of nine amino acids.

of rabbits and mice with high doses of purified HMG1 yields sera with low antibody titers. For this study we have raised antibodies to two different truncated forms of HMG1, to avoid the production of antibodies reactive against the COOH-terminal stretch of negatively charged amino acids. Antibody mAP-bA was obtained by immunization of mice with HMG1/M1-F89, a truncated protein encompassing HMG1 box A. The second antibody was obtained by immunizing a hen with HMG1/M1-K147, a polypeptide containing both boxes A and B of HMG1. The antibodies were purified from egg yolks by affinity chromatography using either immobilized native HMG1/M1-K147 (chIP-AB) or denatured HMG1/M1-K147 (chWB-AB). Finally, we used the original anti-HMG1 chicken antibody (anti-recAtn) raised against recombinant rat amphoterin, which is molecularly identical to HMG1 (Parkkinen et al., 1993).

The four different antibody preparations have different reactivities against HMG1, as shown in Fig. 1 *A*. Reactivity against the native antigens was assessed by dot immunoblots or immunoprecipitation experiments (not shown); reactivity against the denatured antigens was assessed with Western blots (Fig. 1 *B*). In different cell lines (NIH 3T3 fibroblasts, HeLa and Jurkat cells, and mouse Schwann cells) each of the antibodies stained the nucleus (Fig. 1, *C–F* and results not shown). The result was the same with several fixation regimes and permeabilization agents. Significantly, the distribution of HMG1 and of AT-rich heterochromatin (revealed by Hoechst 33258 as spots of brighter fluorescence) do not correlate (Fig. 1, compare *C* with *D*). Confocal microscopy (Fig. 1, *E* and *F*) revealed a diffuse, finely punctate pattern of staining, with little or no HMG1 in the nucleoli.

Tagged HMG1 Protein Is Accumulated in the Cell Nucleus

HMG1 is a member of a protein family including HMG2 and ~80 HMG1-related sequences (Ferrari et al., 1994).

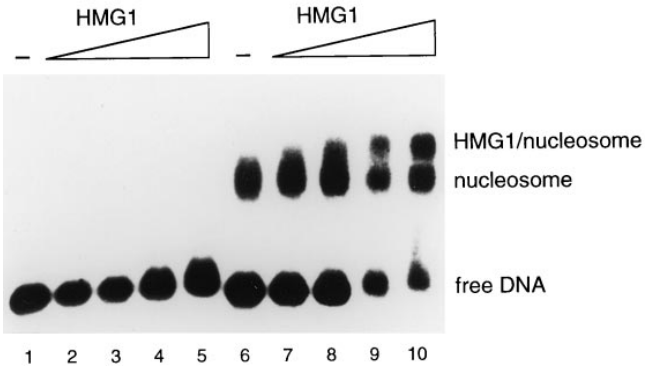

Figure 3. HMG1 binds to reconstituted mononucleosomes. A labeled DNA fragment (176 bp) was assembled into mononucleosomes, incubated with increasing amounts (*0*, *10*, *50*, *100*, and *500* ng) of HMG1 (lanes *6–10*), and electrophoresed on a 0.7% agarose gel. DNA not assembled in nucleosomes was treated similarly for comparison (lanes *1–5*). The bands corresponding to free DNA, to nucleosome particles, and to HMG1–nucleosome complexes are indicated.

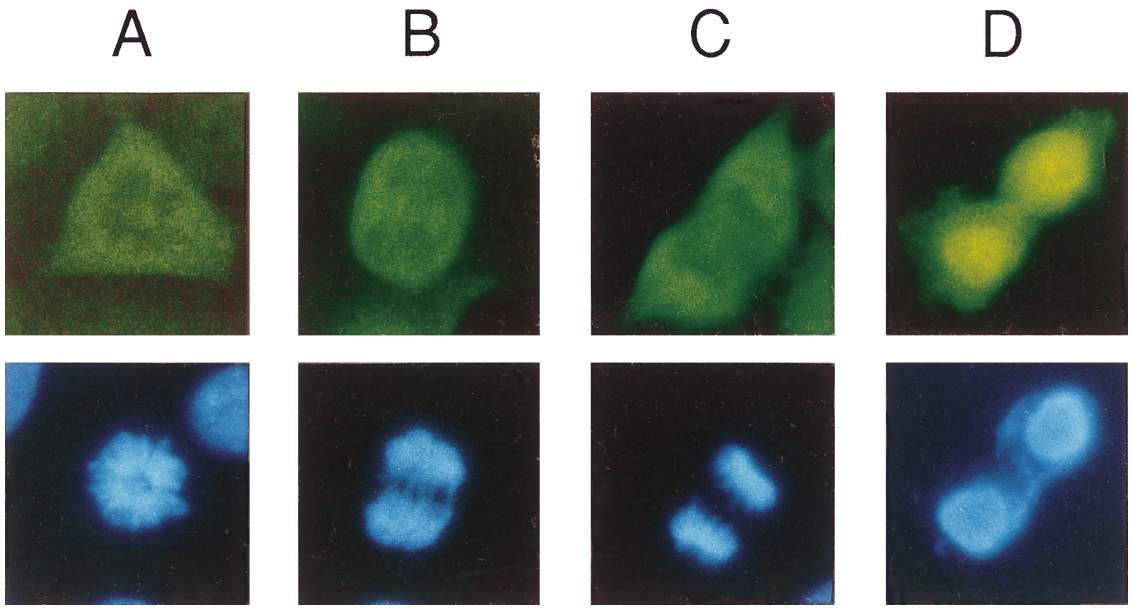

Figure 4. HMG1 protein is not associated to mitotic condensed chromosomes. Dividing NIH 3T3 fibroblasts were fixed and stained for HMG1 with antibody chIP-AB (*top row*, *green fluorescence*) and for DNA with Hoechst 33258 (*bottom row*, *blue fluorescence*). Representative cells at different stages during mitosis: prophase (*A*), metaphase (*B*), anaphase (*C*), and telophase (*D*). After the breakdown of the nuclear membrane, HMG1 diffuses throughout the cytoplasm, and the pattern of green fluorescence corresponds to the shape of the cell. However, fluorescence from HMG1 is clearly reduced in correspondence to the volume occupied by condensed chromosomes (compare *top* and *bottom* images), indicating that HMG1 is not associated with DNA during mitosis. After cell division and the reformation of nuclear membranes (*D*), the majority of HMG1 colocalizes with DNA, but some is still found in the cytoplasm, suggesting that the protein is being concentrated in the nuclei by passage through the nuclear membrane.

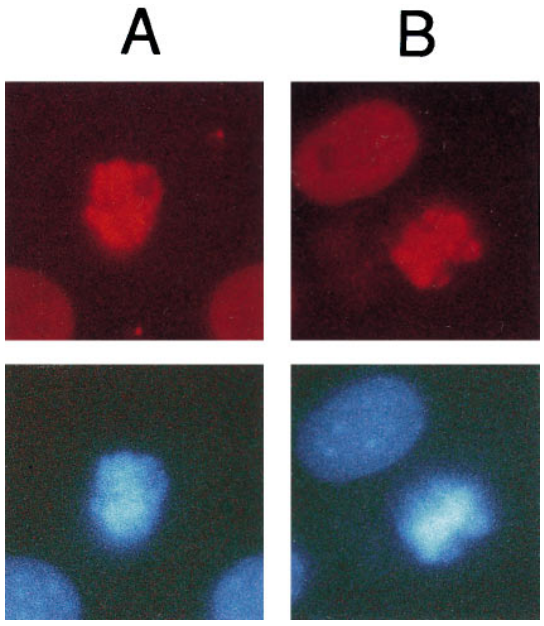

Figure 5. Core histone H2B and linker histone H1 remain associated to condensed chromosomes throughout mitosis. As a control for the mitotic displacement observed with HMG1, dividing NIH 3T3 fibroblasts were fixed and stained for DNA with Hoechst 33258 (*bottom*) and either (*A*) for histone H2B with the monoclonal IgM HBC-7 (*top*) or (*B*) for linker histones with a rabbit polyclonal antibody against histone H1 (*top*).

To exclude the possibility that our antibodies could cross react with proteins closely related to HMG1, we modified the cloned *Hmg1* gene by adding a sequence coding for a nonapeptide from influenza hemagglutinin. The modification does not alter the exon–intron organization of the gene nor its 5′ untranscribed region (Fig. 2 *D*). We obtained several NIH 3T3 clones stably transformed with the tagged gene, one of which (c47) expressed similar amounts of HMG1 and HMG1tag (Fig. 2 *E*). The anti-HA mAb does not stain wild-type NIH 3T3 fibroblasts but brightly stains the nucleus of transformed cells (Fig. 2 *C*).

HMG1 Protein Associates to Nucleosomes In Vitro but Not in Condensed Chromosomes

Nightingale et al. (1996) have recently shown that *Xenopus* HMG1 forms stable complexes with in vitro reconstituted nucleosomes, similar to the complexes formed by histone H1 and its embryonic variant B4. Both HMG1 and B4 associate with linker DNA and protect it from micrococcal nuclease digestion. We confirmed these observations with mammalian HMG1: HMG1 binds very weakly to linear DNA not organized in nucleosomes, whereas it forms complexes with in vitro reconstituted core mononucleosomes (Fig. 3). Nucleosomes lacking linker DNA were unable to bind HMG1 (results not shown).

To further confirm the association of HMG1 with nucleosomes, we then probed whether HMG1 is an integral component of condensed chromosomes, like its proposed *Drosophila* homolog, HMG-D (Ner and Travers, 1994), and histone H1 (Breneman et al., 1993). Unexpectedly, in

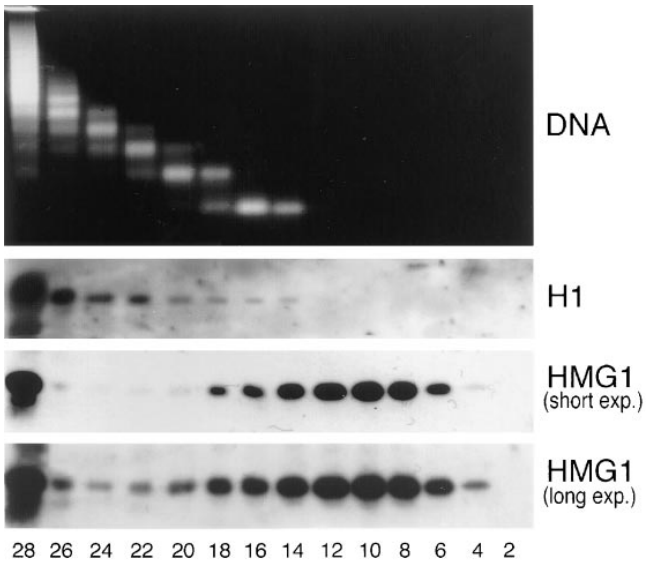

Figure 6. A minor fraction of HMG1 cofractionates with polynucleosomes in sucrose gradients. Nuclei of NIH 3T3 fibroblasts were partially digested with micrococcal nuclease, lysed, and sedimented through a sucrose gradient (see Materials and Methods). Individual fractions were analyzed by Western blotting for the presence of HMG1 and histone H1; DNA was also extracted from the fractions and analyzed on a 2% agarose gel.

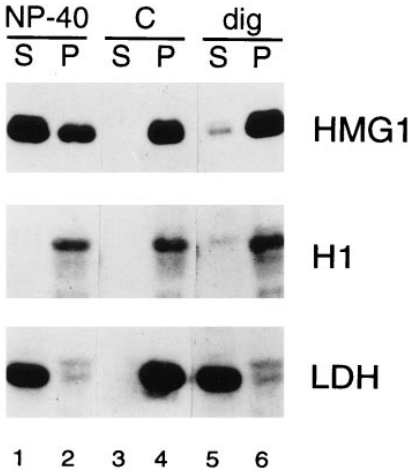

Figure 7. HMG1 leaks out from detergent-permeabilized nuclei of adherent cells, but histone H1 does not. Adherent NIH 3T3 fibroblasts were incubated in buffer with no detergent (lanes *3* and *4*), in buffer containing 0.1% NP-40 (lanes *1* and *2*), or in buffer containing 40 μg/ml digitonin (lanes *5* and *6*). After incubation, the buffer bathing the cells (lanes *1*, *3*, and *5*; *S*, supernatant) and the remnants of permeabilized cells (lanes *2*, *4*, and *6*; *P*, pellet) were analyzed by Western blotting with antibodies against LDH, HMG1, and histone H1. Digitonin selectively permeabilizes the plasma membrane and causes the complete leakage of LDH but not of HMG1 and H1. The faint bands in lanes *2* and *6* do not correspond to LDH, because they have a slightly different molecular weight. NP-40 causes the disruption of all membranes including the nuclear ones: LDH is completely released, as well as ~75% of HMG1 (as determined by densitometric analysis), but H1 remains associated with the DNA. We observed an incomplete release of HMG1 only when we permeabilized cells still attached to their plastic substrate, possibly because HMG1 sticks avidly to secreted glycoproteins (Falciola, L., and M.E. Bianchi, unpublished results).

fibroblasts undergoing mitosis our anti-HMG1 antibodies stained the cytoplasm in a diffuse way (Fig. 4 and results not shown), while the chromosomes appear as dark areas, indicating that HMG1 is displaced from condensed chromatin. As a control for the accessibility of mitotic chromosomes to antibodies, the same cells were stained with the monoclonal IgM antibody HBC-7 directed against histone H2B (Whitfield et al., 1986) and with a polyclonal rabbit antibody directed against histone H1. In contrast to the anti-HMG1 antibodies, the antibodies against core and linker histones brightly stained the condensed chromosomes (Fig. 5).

HMG1 Is Weakly Associated with Nucleosomes in Interphase Chromosomes

The results shown above suggest that the bulk of HMG1 is not associated with mitotic chromatin. To examine HMG1's association to chromatin during interphase, nuclei of NIH 3T3 fibroblasts were partially digested with micrococcal nuclease, and nucleosomal particles were fractionated on sucrose gradients (Fig. 6). Consistent with previous data, histone H1 was found associated with mono- and polynucleosomes. Most of HMG1, however, was recovered at the top of the gradient in fractions that do not contain DNA; only longer exposures of the Western blot revealed a minor amount of HMG1 in the fractions containing polynucleosomes (Fig. 6, lanes 20–26).

Since our results are in contrast to the prevailing view that HMG1 is a structural component of chromatin (van Holde, 1988), we studied the interaction of HMG1 with interphase chromatin by a more gentle technique. Monolayers of fibroblasts were treated either with digitonin, which permeabilizes the plasma membrane exclusively (Diaz and Stahl, 1989), or with NP-40, which permeabilizes all cellular membranes including the nuclear ones. The proteins released in the medium and the cell remnants attached to the plastic surface were analyzed by Western blotting using anti-HMG1 and anti-histone H1, as well as a control antibody against LDH. As shown in Fig. 7 *A*, LDH was almost quantitatively released from the cells by treatment with digitonin, while HMG1 and histone H1 remained contained within the permeabilized cells. On the other hand, treatment with NP-40 released the majority of HMG1, whereas histone H1 remained associated to the cell remnants. The release of HMG1, but not of histone H1, from NP-40–treated cells was also revealed by immunofluorescence. Histone H1 remained within the nucleus in cells fixed either before or after permeabilization, whereas HMG1 was lost from cells permeabilized before fixation (data not shown).

We also compared directly the detergent-mediated release of HMG1 from metaphase and interphase cells. NIH 3T3 fibroblasts were treated with nocodazole, an inhibitor of microtubule assembly; mitotic cells were shaken off the dishes, while nonmitotic cells were detached by mild trypsin digestion (Martinez-Balbás et al., 1995). The two cell populations were then permeabilized with NP-40 and analyzed for protein retention by Western blotting (Fig. 8). In

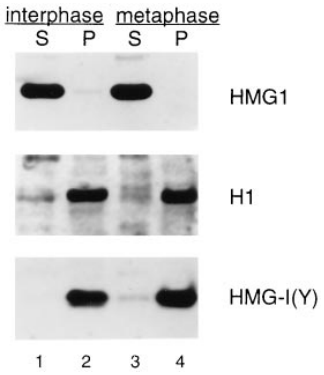

Figure 8. HMG1 leaks out in a similar way from both interphasic and mitotic permeabilized cells. NIH 3T3 fibroblasts were exposed overnight to nocodazole, an inhibitor of microtubule polymerization. Cells that had entered M phase could not proceed further and were detached from their plastic substrate by manual shaking (*metaphase* cells). Cells that remained adherent to the substrate after shaking (*interphase* cells) were detached by treatment with trypsin. The two cell populations were checked for the presence of condensed chromosomes (95% for metaphase cells; 2% for interphase cells). The cell suspensions were then exposed to 0.1% NP-40 and immediately centrifuged. Supernatants (lanes *1* and *3*, *S*) and cell pellets (lanes *2* and *4*, *P*) were analyzed by Western blotting with antibodies against HMG1, histone H1, and protein HMG-I(Y).

both mitotic and nonmitotic fibroblasts, histone H1 is retained in the cell pellets; in contrast, the vast majority of HMG1 is released into the medium. Moreover, HMG-I(Y), a different high mobility group protein which is a component of isolated condensed chromosomes (Saito and Laemmli, 1994) was retained after permeabilization in both mitotic and nonmitotic cells.

These results demonstrate that HMG1 is released as easily from condensed and noncondensed chromosomes. Since HMG1 is apparently excluded from mitotic chromosomes, its association to interphase chromatin is also very weak.

Discussion

By using several purified antibodies, we have clearly established that HMG1 protein is exclusively located in the nucleus in several cell lines. The discrepancy with previous results (for review see Einck and Bustin, 1985) may in part be related to the quality of the antibodies used and to potential cross reaction to HMG1-like molecules. The distribution of epitope-tagged HMG1, however, confirms that HMG1 normally resides in the nucleus.

Confocal microscopy shows that HMG1 is fairly uniformly distributed in the interphase nucleus, with the notable exclusion from nucleoli. We did not see any specific association with (or exclusion from) heterochromatin or speckles containing transcription and splicing factors. This distribution is compatible with a function of HMG1 as a bulk component of chromatin like histone H1, as originally postulated (for review see van Holde, 1988). However, the properties of HMG1 and histone H1 differ in two important respects: displacement from condensed chromosomes and association to chromatin in permeabilized nuclei.

HMG1 is not a component of metaphase chromosomes and diffuses to the cytoplasm when the nuclear membrane is broken down at prometaphase (Fig. 4). By partially solubilizing the membranes of metaphase cells, we can show that condensed chromatin is almost completely devoid of HMG1 but retains core and linker histones as well as the high mobility group protein HMG-I(Y) (Figs. 7 and 8). There is no loss or degradation of HMG1 at metaphase (Fig. 8, compare lanes *1* and *3*), and we are unable to detect any M phase–specific posttranslational modification of the protein (results not shown). Thus, HMG1 is displaced from condensed chromosomes like most transcription factors (Martinez-Balbás et al., 1995), in stark contrast to the *Drosophila* HMG1-like protein HMG-D during embryogenesis (Ner and Travers, 1994).

We have also shown a quantitatively minor association of HMG1 to chromatin even during interphase. HMG1 is almost completely absent from nucleosomal particles obtained by partial digestion of whole nuclei (from Fig. 6 we estimate that a maximum of 2% of the total amount of HMG1 is associated with polynucleosomes) and readily leaks out from detergent-permeabilized interphase cells (Figs. 7 and 8), again in contrast to histone H1 and HMG-I(Y). However, in vitro mammalian HMG1 forms stable complexes with reconstituted nucleosomes (Fig. 3), as previously shown for *Xenopus* HMG1 (Nightingale et al., 1996). This paradox may be only apparent, however. Ura et al. (1996) showed that *Xenopus* HMG1 and histone H1 associate to nucleosomal particles in a way that is qualitatively similar but quantitatively different. Both histone H1 and HMG1 bind to linker DNA, have 1:1 stoichiometry to core nucleosomes, protect chromatosomes from micrococcal nuclease digestion, restrict nucleosome mobility, and repress transcription. In short, there is strong evidence that HMG1 and histone H1 compete for the very same sites in chromatin. However, the relative affinity for these sites is almost 20 times higher for histone H1 than for HMG1 (Ura et al., 1996). Taking into account that in differentiated mammalian cells the molar concentration of histone H1 is at least 10 times higher than that of HMG1 (Einck and Bustin, 1985), one can expect that histone H1 will effectively outcompete HMG1 from linker DNA, as we have shown experimentally.

The outcome of the competition between HMG1-like molecules and histone H1 may be completely reversed during early embryogenesis: *Xenopus* and *Drosophila* embryos have a large stock of maternally inherited HMG1 and HMG-D, respectively (Kleinschmidt et al., 1983; Dimitrov et al., 1993, 1994; Ner and Travers, 1994). Moreover, histone H1 is absent until after the midblastula transition, which occurs late in both organisms. Thus, HMG1 and HMG-D may "play linker histone" until H1 is expressed and takes over. Whether the same applies to mammalian embryogenesis is not obvious, since midblastula transition occurs at the four-cell stage in the mouse (Hogan et al., 1986), and the concentrations of HMG1 and H1 before that stage have not been measured precisely yet.

In conclusion, our results suggest that histone H1 and HMG1 do not play equivalent roles in differentiated mammalian cells like fibroblasts but do not yet exclude that HMG1 might vicariate H1 during mammalian early embryogenesis. The phenomenon of mitotic displacement and the weak association to chromatin disprove a structural role for HMG1 in the packaging of bulk DNA in differentiated cells but are fully compatible with the stable association of HMG1 with a very minor population of nucleosomes or the involvement of HMG1 in transient inter-

actions with chromatin or individual nucleosomes. HMG1 and 2 can ply and mould DNA and have been shown to cooperate with the progesterone receptor, Oct proteins, and HOX gene products in the control of gene expression (Oñate et al., 1994; Zwilling et al., 1995; Zappavigna et al., 1996). We hold the view that the abundance of HMG1 and 2 simply reflects their versatility and usefulness in the construction of a multitude of transient and specialized nucleoprotein complexes, in defiance of the structural rigidity of naked DNA.

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