High-Mobility Group Protein HMG-I Localizes to G/Q- and C-Bands of Human and Mouse Chromosomes

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Abstract. Mammalian metaphase chromosomes can be identified by their characteristic banding pattern when stained with Giemsa dye after brief proteolytic digestion. The resulting G-bands are known to contain regions of DNA enriched in A/T residues and to be the principal location for the L1 (or Kpn 1) family of long interspersed repetitive sequences in human chromosomes. Here we report that antibodies raised against a highly purified and biochemically well characterized nonhistone "High-Mobility Group" protein, HMG-I, specifically localize this protein to the G-bands in mammalian metaphase chromosomes. In some preparations in which chromosomes are highly condensed, HMG-I appears to be located at the centromere and/or telomere regions of mammalian chromosomes as well. To our knowledge, this is the first well-characterized mammalian protein that localizes primarily to G-band regions of chromosomes.

The characteristic banding patterns of mammalian metaphase chromosomes produced by the fluorescent dye quinacrine or by Giemsa staining after limited proteolytic digestion have not only been of immense medical importance in terms of the diagnosis of human genetic diseases and gene mapping but have also radically altered earlier ideas about the basic organization of chromosome structure. Currently, chromosome bands are categorized as fluctuant Giemsa (G-bands), Quinacrine (Q-bands), and Reverse (R-bands), and Constant heterochromatin (C-bands) (Sumner, 1982). These banding patterns correlate with the known "global variations" in nucleotide composition occurring in vertebrate genomic DNA (Ikemura and Aota, 1988) and with the compartmentalization of mammalian chromosomes into large domains of "isochores" enriched in either adenine and thymine (A-T) or guanine and cytosine (G-C) nucleotide sequences (Bernardi et al., 1985).

The G/Q-bands observed in mammalian metaphase chromosomes are preferentially enriched in A-T residues, condense early in mitosis, replicate their DNA late in the S-phase of the cell cycle, seem to be relatively poor in expressed gene sequences, and appear to be the principal location for the L1 (or Kpn 1) family of long interspersed repetitive sequences (LINES) in human chromosomes (see review by Holmquist, 1988).

C-banding, on the other hand, is generally obtained with Giemsa staining after treatment of chromosomes with either mild alkali solution (Sumner, 1982) or after excessive digestion of chromosomes with trypsin (Merrick et al., 1973). Chromosomal C-bands are typically rich in A-T residues and are preferentially located at centromeres, as well as telomeres and nucleolar organizer regions in some organisms (Schweitzer et al., 1987).

In addition to variations in base composition, it is now generally acknowledged that the interactions of particular nonhistone chromatin (NHC) proteins with specific regions or sequences of DNA are also likely to be important determinants of mammalian chromosome staining and/or banding patterns (Babu and Verma, 1987; Sumner, 1982; Cartwright et al., 1982; and Comings, 1978). Indeed, a few examples have been reported of localization of certain NHC proteins to specific chromosome regions such as centromeres (Tharapel and Elgin, 1986; Earnshaw and Rothfield, 1985), telomeres (Gottschling and Zaiken, 1987), the nucleolar organizer region (Guldner et al., 1986), satellite DNA or A-T-rich heterochromatin (Fleischmann et al., 1987; Solomon et al., 1986; Strauss and Varshavsky, 1984; Levinger and Varshavsky, 1982; Alfgemle et al., 1980), to the base of DNA loops on metaphase chromosome scaffolds (Earnshaw et al., 1985), and to lambrush chromosome loops (Roth and Gall, 1989). By comparison, little is known about the mechanism of mammalian chromosome G-banding or the manner in which particular NHC proteins might be involved in the chromatin structure in these regions (Babu and Verma, 1987; Sumner, 1982).

NHC proteins include a group of low-molecular weight (M, <30,000) acid-soluble proteins referred to as the "High-
Mobility Group" (HMG) proteins (Johns, 1982). The precise roles of the various HMG proteins in chromosome structure and function are unknown; however, these proteins may be involved in chromosome or nuclear architecture or gene function (Yang-Yen and Rothblum, 1988; Einck and Bustin, 1985; Reeves, 1984). Previously our laboratory has demonstrated that pure preparations of a newly identified member of the HMG protein family, HMG-I (Lund et al., 1983), specifically binds in vitro to the minor groove of A+T-rich DNA, particularly when such sequences are located in the 3'-untranslated regions of genes (Lehn et al., 1988; Russnak et al., 1988; Reeves et al., 1987; Elton et al., 1987). Additionally, we have recently isolated the mouse and human cDNAs encoding the HMG-I protein (and its isoforms), adding immensely to our understanding of this protein (Johnson et al., 1988, 1989).

Here we demonstrate by indirect immunofluorescence that HMG-I specifically localizes to fluctuant-type G/Q-bands of well-spread human and mouse metaphase chromosomes from disrupted cells and to centromeric and telomeric regions of highly condensed human and mouse metaphase chromosomes inside intact cells.

Materials and Methods

Protein Purification

HMG proteins extracted with 5% perchloric acid from murine ascites cells were separated by ion-pair reverse-phase HPLC (Elton and Reeves, 1986). The proteins, dissolved in solvent A (aqueous 0.1% TFA), were eluted with an 8-50% linear gradient of solvent B (0.1% TFA, 95% acetonitrile, 5% water) in 80 min at a flow rate of 1.0 ml/min. The column effluents from peaks corresponding to HMG-Y and HMG-I were collected, lyophilized, and resuspended in water; their purity was demonstrated by acid-urea polyacrylamide slab gel electrophoresis (Elton and Reeves, 1986).

Antibody Production and Western Blot Analysis

Rabbits were initially injected with pure HMG-I protein (0.25 mg) cross-linked to Limulus hemocyanin (0.25 mg; Sigma Chemical Co., St. Louis, MO) with glutaraldehyde. The cross-linked proteins were initially injected subcutaneously with complete Freund's adjuvant and thereafter, the rabbits were boosted at regular intervals with pure HMG-I protein alone (~0.1 mg per injection). The anti-HMG-I IgG fraction was separated from whole sera with a protein A column (Miller and Stone, 1978) and these IgGs were used for all experiments. The antibody titer and specificity was determined by ELISA (Voller and Bidwell, 1986). The antibody specificity was further demonstrated by Western blot analysis as described by Towbin et al. (1979). Briefly, protein samples were extracted with 0.35 M NaCl from isolated nuclei and separated, along with marker protein standards, by SDS/PAGE on 15% polyacrylamide gels. The proteins were electro-transferred to a nitrocellulose membrane and reacted with anti-HMG-I IgGs (1:100 dilution). The rabbit HMG-I antibody was localized on the membrane by reaction with peroxidase-conjugated goat anti-rabbit IgGs (Towbin et al., 1979).

Chromosome Preparation

Mouse Friend erythroleukemic cell line 745A and human erythroleukemic cell line K562 were maintained in RPMI media containing 5% FCS at 7% CO2. These cell lines were chosen because we had previously demonstrated that they express significant levels of the HMG-I protein (Johnston et al., 1989) and because they are rapidly proliferating and consequently have a high mitotic index in mid-log phase cultures. Cells were collected at mid-log phase, suspended in buffer A (Palmer and Margolis, 1985), and spun for 8 min at 1,000 rpm in a cytogenetifupe (Shandon Southern Instruments, Sewickley, PA). It was necessary to spin unfixed chromosomes onto slides to preserve protein-DNA interactions and protein integrity. The cells were immediately fixed with Bouin's fluid (Polysciences, Inc., Warrington, PA), rinsed with distilled water, dehydrated in 70% ethanol, rinsed with distilled water again, and soaked in 1% BSA in PBS (PBS/BSA), pH = 7.5 (5 min at each step).

Indirect Immunofluorescence

Cells fixed with Bouin's fluid were incubated overnight at 4°C with either the anti-HMG-I IgGs, diluted 1:100 in PBS/BSA or, in control experiments, with mouse antihistones in ascites fluid (Chemicon International, Inc., El Segundo, CA) diluted 1:500 in PBS/BSA. Slides were then washed with PBS/BSA and incubated at room temperature for 2 h with biotinylated goat anti-rabbit IgGs (Sigma Chemical Co.) or biotinylated goat anti-mouse IgGs (Chemicon International, Inc.) diluted 1:40 in PBS/BSA. Cells were washed with PBS/BSA followed by PBS alone and then one of two different indirect immunofluorescence protocols was used. (a) Cells were incubated at room temperature for 30 min with avidin-FITC (EY Laboratories, San Mateo, CA) diluted 1:10 in PBS, washed for 30 min with PBS, and mounted in glycerol containing n-propyl gallate as an antiquenching agent (Giloh and Sedat, 1982) and 0.1 μg/ml propidium iodide as a generalized DNA counterstain (see Fig. 3, C, E, and G-I). (b) Cells were incubated at room temperature for 1 h with fluorescein-avidin DCS (Vector Laboratories, Burlingame, CA) diluted 1:800 with PBS/BSA and then washed for 30 min with PBS/BSA. This was followed by a 1-h room temperature incubation with anti-avidin D (Vector Laboratories) diluted 1:100 with PBS/BSA. After washing for 30 min with PBS/BSA, the cells were incubated with a 1:400 dilution of fluorescein-avidin DCS in PBS/BSA for one hour and washed three times with PBS/BSA. Cells were counterstained with 2.5 μg/ml propidium iodide in PBS for 15 s, rinsed for 2 min in McIlwain's buffer at pH = 7.5 and mounted as described above (see Fig. 3, A, B, D, and F).

G-Banding

Human K562 cells were treated with 0.025 μg/ml colcemid for 90 min, incubated with 0.56% KCl at 37°C for 30 min, and fixed in 3:1 methanol acetic acid. Slides were prepared by the wet slide method (Hansen, 1981), aged 20 min at 90°C, treated with 0.025% trypsin in 0.9% NaCl for 45 s, rinsed twice in 0.9% NaCl, stained in 1:4 Wright stain/pHydrion buffer, pH = 6.8 for 90 s, rinsed with distilled water and air-dried.

Chromosome Identification

The immunofluorescent human chromosomes were identified based on their G-banding-like patterns and not their morphology. Some chromosomes were difficult to identify because the karyotype of the human K562 cell line is grossly rearranged. Also, spinning unfixed chromosomes onto slides can lead to chromosome distortion and an unconventional appearance of some chromosomes as compared with air-drying methanol acetic acid-fixed chromosomes onto slides.

Photomicrography

Cells were photographed with a Nikon microscope equipped with epifluorescence. Color photomicrographs were taken with Kodak Ektachrome 160 color slide film and reproduced with Type C positive printing (Fig. 3, C and E) or with Fujicolor Super HR 400 print film at ASA settings of 160 or 200 (Fig. 3, A, B, D, F, and G-I). Black-and-white photomicrographs were taken with Kodak Technical Pan film (Fig. 4 A) or black-and-white prints were made from negatives of color slides shot on Kodak Plus X film (Figs. 4 B and 5).

Results

Protein Purification and Antibody Specificity

Precise immunolocalization of proteins within cells requires the use of specific antibody preparations. Biochemically homogeneous preparations of murine HMG-I protein (Mr = 11,700) were isolated by preparative reverse-phase HPLC (Fig. 1) and used to elicit polyclonal antibodies in rabbits. Western blot analysis revealed that the HMG-I antibody was specific for the HMG-I (and its isoform HMG-Y) protein in total nuclear protein extracts from either murine or human
Figure 1. HMG-I and HMG-Y are purified to homogeneity using reverse-phase HPLC. The total protein extract before HPLC separation (20 μg) is shown in the left lane; HPLC-purified HMG-Y (5 μg) is shown in the middle lane; and HPLC-purified HMG-I (5 μg) is shown in the right lane. The identities of these purified protein fractions were further confirmed by partial peptide sequence analysis (Johnson et al., 1989; Lehn et al., 1988).

Figure 2. HMG-I antibody specificity is confirmed by Western blot analysis. (A) Coomassie blue-stained protein gel. (B) Western blot incubated with anti-HMG-I IgGs. Lanes: 1, mouse HMG-17 protein (2 μg); 2, mouse HMG-I protein (2 μg); 3, mouse HMG-Y protein (2 μg); 4, human K562 erythroleukemia cell nuclear proteins; 5, human HUT-78 leukemia cell nuclear proteins; 6, mouse Friend erythroleukemia cell nuclear proteins; arrowheads, position of the HMG-I proteins.

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The nonrandom distribution of the HMG-I protein into specific bands on human metaphase chromosomes from disrupted cells contrasts with the random distribution of histone proteins on similarly prepared metaphase chromosomes. Indirect immunofluorescence with antihistone antibody resulted in a uniform distribution of fluorescence over chromosomes (Fig. 3 D). It is therefore unlikely that our fixation protocol either selectively extracts proteins from particular areas of chromosomes or preferentially affects accessibility of antibodies to the R-band regions of such chromosomes. Such a uniform immunolocalization of histone proteins on metaphase chromosomes is consistent with the results of Bustin et al. (1976).

In addition to the experiments with the antihistone anti-

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Figure 3. HMG-I localizes to G-band regions of human and mouse metaphase chromosomes and to the nucleus throughout mitosis in human cells. (A, B, and C) Human chromosomes from disrupted cells appear banded after an indirect immunofluorescence protocol involving treatment of chromosomes with polyclonal anti-HMG-I, biotinylated goat anti–rabbit IgGs, and avidin-FITC. The indicated chromosomes were identified based on their characteristic G-banding-like immunofluorescent patterns. Identification was greatly facilitated by using the fluorescent red DNA counterstain propidium iodide. In B, a nucleolus organizer region devoid of specific immunofluorescence. Note that HMG-I is detected in the satellite region of this chromosome (S). (Inset) Chromosomes from a separate mitotic cell. (D) Human chromosomes from a disrupted cell appear uniformly stained after a similar indirect immunofluorescence protocol involving monoclonal anti–histone, biotinylated goat anti–mouse IgGs, and avidin-FITC. These chromosomes were also counterstained with propidium iodide. (E and F) Mouse Friend cell prometaphase chromosomes inside an intact cell (E) and midmetaphase chromosomes from a disrupted cell (F) appear banded after indirect immunofluorescence with anti-HMG-I as described above. (G) Human K562 cell midmetaphase chromosomes inside an intact cell display specific anti-HMG-I immunofluorescence in the centromere regions (small arrowheads) and telomere regions (large arrowheads) of some chromosomes. (H) Human K562 cell anaphase chromosomes display a dispersed, punctate pattern of anti-HMG-I immunofluorescence. (I) Human K562 interphase cells display specific anti-HMG-I immunofluorescence primarily in the nucleus. Bar, 5 μm.

Figure 4. Conventional G-banding patterns correspond with the HMG-I antibody immunofluorescence patterns on human chromosomes. (A) Human chromosomes G-banded by conventional trypsin/Wright stain technique. (B) Pair-wise comparison of representative human chromosomes. Immunofluorescent chromosomes on the left were taken from black-and-white photos of the counterstained chromosomes in Fig. 3 C. Trypsin G-banded chromosomes on the right are from the cell depicted in Fig. 4 A. Bar, 10 μm.
body, the results of the following experiments also support the specific localization of HMG-I on mammalian metaphase chromosomes from disrupted cells. (a) Normal rabbit serum (or purified IgGs) did not specifically label either human or mouse metaphase chromosomes when used in indirect immunofluorescent assays (Fig. 5 A). (b) Pure HMG-I protein effectively competed for binding with the HMG-I antibody in immunofluorescent localization experiments. When increasing concentrations of pure murine HMG-I protein were added to the antibody preparation at the time of initial reaction with mammalian chromosomes, the amount of resulting specific immunofluorescence detected in chromosomes was greatly attenuated and decreased as a function of competitor protein concentration (Fig. 5, B–D). (c) With increasing times of digestion of human chromosomes with trypsin, under conditions normally used to reveal mammalian chromosome G-banding patterns, there was a progressive and corresponding loss of chromosomal immunofluorescence (data not shown).

We conclude from these results that the HMG-I antibody is specifically detecting HMG-I protein in the G-band regions of mammalian metaphase chromosomes and that this NHC protein is either altered or destroyed as a result of the brief trypsin treatment (see below for discussion).

**HMG-I Localizes to Centromere and Telomere Regions of Metaphase Chromosomes inside Intact Cells**

In addition to G-bands, the HMG-I antibody can specifically bind to the centromere and telomere regions of mouse and human chromosomes when chromosomes remain inside intact cells. As described above for chromosomes from disrupted cells, there is no specific immunofluorescence on chromosomes inside intact cells when normal rabbit IgGs are used in indirect immunofluorescence experiments and specific immunofluorescence of centromeric and telomeric regions can be abolished in competition experiments with purified HMG-I protein (data not shown). In Fig. 3, E and G it is apparent that HMG-I can be distributed differently on different metaphase chromosomes within the same intact cell. HMG-I localizes to telomeric and centromeric regions of some chromosomes, and only the telomeric regions or only the centromeric regions of others. Some chromosomes display intercalary bands as well. This variation is consistently observed when mitotic cells remain intact during the centrifugation procedure.

We interpret the variable antibody binding seen in these types of preparations to be the result of the high degree of

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**Figure 5.** Excess murine HMG-I competes with human chromosomal HMG-I for binding to the HMG-I antibody. (A) Incubation of K562 cells with normal rabbit IgGs (diluted 1:100) does not result in nuclear fluorescence after treatment with biotinylated goat anti-rabbit IgGs and avidin-FITC. (B) Treatment of chromosomes with the HMG-I antibody results in typical bright fluorescent patterns of transverse bands. (C) Addition of 1.0 μg reverse-phase HPLC-purified HMG-I with the anti-HMG-I IgGs at the time of incubation results in noticeable attenuation or reduction in chromosomal immunofluorescence. (D) Addition of 20 μg of RP-HPLC purified HMG-I with the anti-HMG-I IgGs at the time of incubation results in almost total attenuation or loss of chromosomal immunofluorescence. Bar, 10 μm.
chromosome compaction in intact mitotic cells; the HMG-I antibody has limited access to some chromosome regions in intact cells that are exposed when chromosomes are freed from the cell. Three lines of evidence lead us to this assessment. (a) Prometaphase chromosome states, such as those depicted in Fig. 3 E, are less condensed and display more intercalary bands than metaphase chromosomes such as those depicted in Fig. 3 G. (b) The overall immunofluorescence is diminished and becomes more punctate as cells move into anaphase (Fig. 3 H) and telophase. At these stages chromosomes are more condensed than at midmetaphase. (c) The histone antibody also has limited access to some chromosome regions in intact mitotic cells (data not shown).

We suggest that the compaction of chromatin in intact mitotic cells permits immunolocalization of HMG-I to centromere and telomere regions of chromosomes and that this specific immunofluorescence cannot be similarly visualized in less condensed chromosomes from disrupted cells.

**HMG-I Localizes to Nuclei of Interphase Cells**

The principle location of HMG-I in interphase cells from mid-log phase cultures of both mouse Friend and human K562 erythroleukemic cells appears to be the nucleus (see Fig. 3 I). Very little immunofluorescence is detected in the cytoplasm of these cells. HMG-I is not excluded from nor is it specifically and exclusively localized to the nucleoli of interphase cells.

**Discussion**

We have demonstrated by indirect immunofluorescence that HMG-I localizes to A-T-rich G-band regions of human and mouse chromosomes. HMG-I antibody binding to G-bands can be abolished by a brief trypsin treatment comparable to that used in G-banding protocols. Early EM studies suggested that there is a rearrangement of chromatin fibers as a result of trypsin G-banding (Burkholder, 1974). Comings (1978) suggested that, as a result of trypsin treatment of mammalian chromosomes, DNA in the G-band regions is more accessible to the thiazin dyes present in Giemsa stain than in the DNA in the interband regions. Brief trypsin digestion may preferentially remove HMG-I from G-band regions of chromosomes, thus altering the structure in these regions and allowing more ready access of the thiazin dyes in Giemsa stain to the DNA in these regions.

In *Drosophila*, several NHC proteins with certain biochemical similarities to the mammalian HMG proteins have been localized to A-T-rich regions of polytene chromosomes. One such protein, D1 (MW = 50,000), is rich in both basic and acidic amino acids and can be extracted from nuclei with 0.35 M NaCl or solubilized with perchloric acid (Levinger and Varshavsky, 1982; Alfageme et al., 1980). Another such protein, antigen CIA9 (molecular weight = 18,101), is also very hydrophilic and has a high predicted helical content, similar to HMG proteins HMGs -1 and -2 (Tharappel and Elgin, 1986). Both of these *Drosophila* proteins localize to heterochromatin regions of polytene chromosomes (including centromeric and some intercalary heterochromatin [Tharappel and Elgin, 1986] and to A-T-rich regions that stain brightly with quinacrine [Levinger and Varshavsky, 1982; Alfageme et al., 1980]). Proposed functions for the D1 and CIA9 proteins include repression of gene function and compaction of A-T-rich heterochromatin.

Recently, Ashley et al., (1989) reported that D1 shares some sequence homology with HMG-I, particularly in the proposed binding motifs. Peptide sequence analysis (Johnson et al., 1988, 1989; Lehn et al., 1988; Lund et al., 1987), as well as nucleotide sequence analysis (Johnson et al., 1988, 1989), of the human and murine HMG-I and HMG-Y proteins and their corresponding cDNAs reveal that each of these NHC proteins contain three copies of a highly conserved palindromic amino acid sequence motif (Pro-Arg-Gly-Arg-Pro) that may be involved in DNA binding. The larger *Drosophila* D1 protein has a number of similar motifs, all of which contain the Gly-Arg-Pro tripeptide.

It has been observed that these conserved peptide motifs have structural similarity to the A-T-rich binding antitumor and antiviral drugs distamycin A and netropsin (Johnson et al., 1988, 1989; Lehn et al., 1988; Lund et al., 1987). When mouse cells are grown in the presence of distamycin A or a similar A-T binding ligand, Hoechst 33258, mitotic chromatin condensation is inhibited, especially in the centromeric region (Radic et al., 1987; and Lica et al., 1986). Hoechst 33258 treated mouse chromosomes are also devoid of kinetochores, a possible explanation being that the dye displaces a protein or proteins responsible for a particular higher order structure of the centromeric heterochromatin necessary for kinetochore assembly (Lica et al., 1986). We know that Hoechst 33258 and distamycin A will effectively compete with purified HMG-I for binding to A-T-rich mouse satellite DNA sequences (Radic et al., 1989, manuscript submitted for publication). We are currently investigating the possibility that HMG-I may be the centromeric protein displaced by in vivo treatment of cells with these A-T binding drugs.

**Conclusions**

In this report we present evidence that a biochemically well-characterized nuclear NHC glycoprotein, HMG-I, is specifically localized to G/Q-bands, centromeres, and telomeres of mammalian metaphase chromosomes. Previous work (Johnson et al., 1988, 1989) has demonstrated that the HMG-I and HMG-Y proteins and mRNAs are usually detectable in high concentrations only in mammalian cells actively engaged in cell proliferation and mitosis. The present finding, that during metaphase the HMG-I proteins are specifically associated with particular regions of chromosomes, adds further support to the idea that they may be actively involved in dynamic changes in chromatin structure occurring during the cell and chromosome condensation cycle. The availability of purified HMG-I and HMG-Y proteins, antibodies, and cDNA clones should prove useful in further studies involving expression of the HMG-I gene in different cell types and at different stages of the cell cycle, as well as in studies aimed at elucidating the roles that these unusual NHC proteins play in the structure and function of mammalian chromosomes.

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