High Mobility Group Proteins: Abundance, Turnover, and Relationship to Transcriptionally Active Chromatin†

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ABSTRACT: We have measured the abundance of high mobility group (HMG) proteins 14 and 17 in HeLa cell chromatin and their fractionation with respect to transcriptionally active sequences. HMG protein 17 constitutes 10–20% of the mass of an individual core histone; HMG 14 is approximately one-tenth the mass of HMG 17. The enrichment of HMG proteins, relative to bulk chromatin, is less than 2-fold in the chromatin fraction enriched 6-fold in active sequences. The digestion characteristics of HMG nucleosomes indicate that they are interspersed with H1 nucleosomes and other monomer species. The HMG monomers are quite resistant to degradation by micrococcal nuclease and can be resolved as distinct nucleoprotein entities after trimming of the DNA to core length. Turnover measurements showed that HMG proteins 14 and 17 are stable for at least 24 h. When nucleosome monomers are reconstituted with a 0.35 M NaCl nuclear protein extract, each nucleosome subtype can be reconstituted; however, this is a function of both the amount of extract added and the DNA length of the nucleosomes. When the kinetics of reconstitution of bulk vs. coding sequences were measured with cDNA, there was no significant enrichment of active sequences in the HMG-containing mononucleosomes of HeLa cells at any ratio of extract to monomer employed. In Friend cells, the abundance of sequences among mononucleosome species was the same for the transcribed β-major globin gene, a transcriptionally inactive embryonic globin, and an inactive immunoglobulin gene. There was little correlation of HMG content with transcriptionally active chromatin, either native or reconstituted.

The high mobility group (HMG) proteins have been the subject of recent interest, due to their reported role as agents of transcriptional activation of nucleosomes (Weisbrod & Weintraub, 1979, 1981; Weisbrod et al., 1980; Gazit et al., 1980; Levy et al., 1977, 1979; Gabrielli et al., 1981). Although the five HMG proteins (HMGs 1, 2, E, 14, and 17) are considered as a category due to their similar electrophoretic and fractionation properties (Johns, 1964; Goodwin et al., 1978), functional similarities among these proteins are not certain.

The HMG proteins can be isolated as nucleosomal components (Goodwin et al., 1977; Mathew et al., 1979; Albright et al., 1980; Jackson et al., 1979; Annunziato et al., 1981) and can be reconstituted with mononucleosomes (Goodwin et al., 1977, Albright et al., 1980; Mardian et al., 1980; Sandeen et al., 1980). HMG proteins 14 and 17 have two binding sites on the nucleosome core (Albright et al., 1980; Mardian et al., 1980), while HMG proteins 1 and 2 have been implicated in binding the linker DNA (Levy et al., 1977; Jackson et al., 1979; Peters et al., 1979).

Conflicting evidence has arisen concerning the preferential solubilization of HMG proteins from transcriptionally active nucleosomes during digestion of nuclei with DNase I. According to one report (Weisbrod & Weintraub, 1979), HMGs 14 and 17 are selectively displaced to the medium during DNase I digestion of nuclei to 10–20% acid solubility. Other studies with DNase I have shown no such release of HMG 14 or 17 under similar conditions (Vidali et al., 1977; Goodwin & Johns, 1978; Levy & Dixon, 1978).

The HMG proteins, and virtually all non-histone chromatin proteins as well, can be extracted from nuclei or chromatin by 0.35 M NaCl. Salt extraction is accompanied by a loss of preferential DNase I sensitivity of active chromatin (Weisbrod & Weintraub, 1979; Weisbrod et al., 1980; Gazit et al., 1980); this sensitivity can be restored by reconstitution of salt-stripped nuclei or chromatin with the salt-eluted proteins (Weisbrod & Weintraub, 1979) or with purified HMG proteins 14 and 17 (Weisbrod et al., 1980; Gazit et al., 1980). In one report, however, the HMG-reconstituted nucleosomes were rendered more DNase I resistant (Sandeen et al., 1980).

In reconstitution experiments with mononucleosomes, the HMG proteins have been reported to reconstitute preferentially with nucleosomes containing transcribed DNA (Weisbrod & Weintraub, 1979, 1981; Sandeen et al., 1980; Weisbrod, 1982), despite the compositional and chemical similarity between monomers derived from either active or inactive chromatin (Weisbrod & Weintraub, 1981; Albright et al., 1980; Mardian et al., 1980; Noll & Kornberg, 1977; Reudelhuber et al., 1980; Weisbrod, 1982).

We have examined relationships between HMG proteins and transcriptionally active chromatin from HeLa and Friend erythroleukemia cells in an effort to understand the affinity of HMG proteins for transcriptionally competent nucleosomes, in light of the evidence that inactive as well as active nucleosomes possess two HMG binding sites (Albright et al., 1980; Mardian et al., 1980; Sandeen et al., 1980). We have quantitated the amount of HMG proteins in whole chromatin and in chromatin fractions enriched for transcriptionally active sequences. In addition, we have examined the distribution of active and inactive sequences among mononucleosome species known to contain or to be devoid of HMG proteins, as well as the selectivity of reconstitution of salt-stripped nucleosomes. The results of these experiments do not encourage the concept that HMG proteins are the primary agents of the transcriptionally active state of chromatin.

Experimental Procedures

Methods. HeLa cells were grown in suspension culture, and nuclei were isolated by homogenization of cells in low ionic strength buffer as described (Annunziato et al., 1981; Seale,

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1981a). Digestion of nuclei was performed by adding $CaCl_2$ to 10^{-4} M, and micrococcal nuclease (Sigma) at 1 unit/10 A_{260} units of nuclei prewarmed to 37 °C. Digestion was terminated by addition of ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) to 1 mM. Nuclei were collected by centrifugation for 5 min in an Eppendorf microfuge at 4 °C. The first supernatant (S1) was removed, and the nuclei were suspended in 2 mM ethylenediaminetetraacetic acid (EDTA) for 16 h in order to release the soluble chromatin. The solubilized chromatin (S2) was clarified by centrifugation for 5 min in an Eppendorf microfuge and removed from the final insoluble nuclear residue (P). Yields of DNA in these three fractions were 5–10% S1, 70–80% S2, and 10–20% P, depending on the extent of digestion. In most preparations, digestion was to 5% acid solubility.

Electrophoresis. Nuclease-solubilized chromatin was analyzed by electrophoresis in agarose–acrylamide (DNP) gels (Todd & Garrard, 1979; Annunziato et al., 1981). Proteins were examined in sodium dodecyl sulfate (SDS)–polyacrylamide gels (Thomas & Kornberg, 1975) by staining and fluorography (Laskey & Mills, 1975). DNA was purified by treatment with proteinase K in 0.5% SDS, followed by phenol extraction and ethanol precipitation, and subjected to electrophoresis in 4% polyacrylamide gels (Loening, 1967).

DNP gels were blotted in 20 × SSC (Southern, 1975) and hybridized to [^{32}P]cDNA prepared against poly(A+) mRNA purified from HeLa polyribosomes by oligo(dT)–cellulose chromatography (Aviv & Leder, 1972). In the experiments with Friend cells, plasmids were nick translated with [^{32}P]-dCTP to a specific activity of 5×10^7 cpm/μg (Rigby et al., 1977). The plasmids employed were cloned cDNAs specific for the β-major globin gene (Rougeon & Mach, 1976), the embryonic globin gene εY3 (Fantoni et al., 1979), or an immunoglobulin light κ chain (Davis et al., 1980).

HMG proteins were isolated from a 0.4 N H_2SO_4 extract of nuclei, or of chromatin fractions (above), by sequential trichloroacetic acid (Cl_3CCOOH) precipitations at 2%, 10%, and 25% (Goodwin et al., 1978); the 25% Cl_3CCOOH precipitate contained HMG proteins 14 and 17.

Reconstitution. Nuclei were isolated and extracted with either 0.35 or 0.45 M NaCl to provide a source of salt-dissociable proteins. Soluble (S2) chromatin was prepared by nuclease digestion as above and depleted of accessory proteins by washing in 0.45 M NaCl (Seale, 1981a,b). The nuclear protein extracts were added in sequential amounts to stripped chromatin and reconstituted by step dialysis (Nelson et al., 1979).

For reconstitution of individual monomer species, a preparative DNP gel was run; individual mononucleosome species were located by ultraviolet (UV) shadowing and excised. The excised gel segments were placed in tubes closed at one end with dialysis bags and subjected to electrophoresis at 4 °C for sufficient time to elute the nucleoproteins (4–5 h). Samples were concentrated at 4 °C against dry Sephadex. Lyophilization, or concentration to dryness, served to dissociate the DNP complexes, as did exposure to ethidium bromide.

Results

Abundance of HMGs 14 and 17 in Chromatin. During the course of experiments concerning nucleosomes bound with HMG proteins (Annunziato et al., 1981), we sought to quantitate the amount of HMGs 14 and 17 as part of a characterization of the putative role of these accessory nucleosomal proteins in the transcriptional activation of nucleosomes. The HMG proteins 14 and 17 are barely detectable in gels by staining acid-extracted chromatin proteins. For this reason, we exploited the increased sensitivity of fluorography to facilitate detection and quantitation of HMG proteins, relative to core histones. In order for measurements by fluorography to be accurate, it was necessary to establish the turnover characteristics of HMG proteins, i.e., that incorporated radioactivity would be stable, and thus would be an accurate reflection of mass. Cells were incubated for 30 min in [^{3}H]lysine, and sampling was begun 10 min after addition of excess unlabeled lysine. Previous experiments have shown the chase conditions to be fully effective (Seale, 1981a), and the 10-min lapse between addition of lysine and initiation of sampling was included so as to allow newly synthesized proteins to become incorporated into chromatin (Seale, 1981a,b).

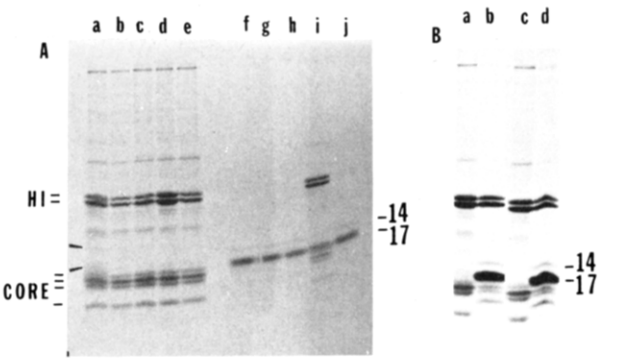

FIGURE 1: (A) Metabolic stability of HMG proteins 14 and 17. Cells were labeled for 30 min with [^{3}H]lysine and then incubated in non-radioactive lysine excess (text). Nuclei were prepared at 0, 1, 2, 3.5, and 5 h and extracted with 0.4 N H_2SO_4. The acid extract was sequentially precipitated with 2%, 10%, and 25% Cl_3CCOOH. Lanes a–e are 0.1 volume of each of the respective 10% Cl_3CCOOH precipitates, and lanes f–j are the entire 25% Cl_3CCOOH precipitates, subjected to electrophoresis in SDS–polyacrylamide gels. A fluorograph is shown. The 2% Cl_3CCOOH precipitates (not shown) contained an equivalent amount of histones as the 10% Cl_3CCOOH precipitate. (B) Cells were labeled as in (A) and chased for 16 h. (Lanes a and c) 10% Cl_3CCOOH-precipitable proteins, 0 and 16 h, respectively; (lanes b and d) 25% Cl_3CCOOH precipitates, 0 and 16 h, respectively. The amount of 10% Cl_3CCOOH precipitates is half that, relative to the 25% Cl_3CCOOH precipitate, used in panel A.

Figure 1A shows that during a 5-h chase period, the HMG proteins 14 and 17 retained the initial level of radioactivity and thus were stable. Figure 1B shows that no significant loss of radioactivity from HMGs 14 and 17 occurred during a 16-h chase. The HMG protein load relative to the core histones in panel B is twice that in panel A. The histone contaminants introduced in Figure 1A, lane i, serve to delineate the electrophoretic position of H3 from that of HMG 17. Analysis of these fractions in acid–urea gels (not shown) confirmed the identity of HMGs 14 and 17, as well as their abundance as indicated in SDS gels. The HMG proteins 14 and 17 were thus stable and suitable for labeling in vivo for one generation for quantitation relative to core histone abundance by fluorography.

It may be noted in Figure 1A, lane a, that rapidly turning over proteins with similar electrophoretic mobilities to HMG proteins 14 and 17 are present (arrows). These were not HMG proteins due to (1) slightly different molecular weights, (2) precipitability in 10% Cl_3CCOOH, and (3) incorporation of [^{35}S]methionine (HMGs 14 and 17 contain no methionine).

After cells were uniformly labeled with [^{3}H]lysine, nuclei were isolated and extracted with 0.4 N H_2SO_4. The acid extract was then adjusted to 10% Cl_3CCOOH in order to precipitate histones and other acid-soluble proteins and then to 25% Cl_3CCOOH in order to precipitate the HMG proteins 14 and 17. The entire HMG protein sample was applied to

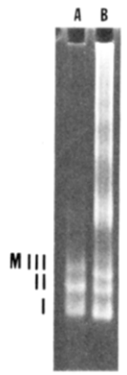

FIGURE 2: Soluble chromatin fractions obtained by the procedure of Bloom & Anderson (1978) subjected to electrophoresis in an agarose–acrylamide (DNP) gel, stained with ethidium bromide, and photographed. (Lane A) S1 chromatin; (lane B) S2 chromatin.

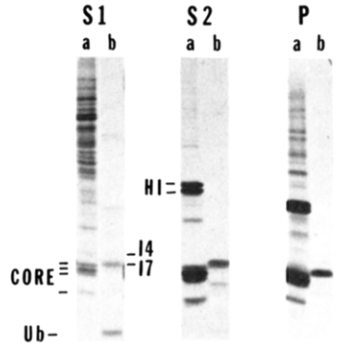

FIGURE 3: Abundance of HMG proteins in fractionated chromatin. Cells were incubated in [^{3}H]lysine for 18 h. Each fraction obtained as in Figure 2, including the insoluble pellet, was acid extracted, and the acid extract was fractionated with Cl_3CCOOH as in Figure 1. Each fraction is indicated in the figure: (lanes a) 10% Cl_3CCOOH-precipitable proteins; (lanes b) 25% Cl_3CCOOH-precipitable proteins. Ub, ubiquitin.

the gel as well as a wide concentration range of the 10% Cl_3CCOOH precipitated histones in order to construct a standard curve, against which the HMG band intensities were compared. The values were then corrected for lysine content [core histones, 10% (Isenberg, 1979); HMGs 14 and 17, 20% (Walker et al., 1977, 1979)]. We obtained a value of 10–20% HMG 17 per core histone, or 2.5–5% of the total core histone mass. The amount of HMG 14 is approximately 0.1 that of HMG 17. These values correspond closely to those reported by others (Goodwin et al., 1973; Vidali et al., 1977; Gabrielli et al., 1981).

HMG Protein Distribution among Chromatin Fractions. The chromatin fractionation procedure of Bloom & Anderson (1978) was employed in the investigation of HMG enrichment in active chromatin since a fraction that is 5-fold enriched in active sequences is readily obtained. This fraction (S1) is the supernatant that results from micrococcal nuclease digestion, followed by sedimentation of nuclei. Under the usual digestion conditions employed in this report, digestion to ~5% acid solubility yielded 5–10% of the remaining DNA as mononucleosomes (Figure 2, lane A). A second supernatant (S2) is obtained by solubilization of the digested chromatin in 2 mM EDTA and contains 70–80% of the DNA (Figure 2, lane B). Fraction S1 contains no H1 (see Figure 3); the electrophoretic mobility difference of the HMG monomer, IIIB, of the S1 fraction from that of the H1 monomer, IIIA, of the S2 fraction is evident in Figure 2 (Albright et al., 1980; Annunziato et al., 1981). The remaining insoluble pellet contains 10–20% of the total DNA, requires high ionic strength (0.4–0.6 M NaCl) for solubilization, and thus is not suitable for analysis as native chromatin in nucleoprotein gels.

In order to account for all HMG protein, each of the three chromatin fractions was acid extracted, and the extracts were further fractionated into histones plus acid-soluble NHP (2%, 10% Cl_3CCOOH) and HMG proteins 14 and 17 (25% Cl_3CCOOH). The proteins from [^{3}H]lysine-labeled cells were resolved in SDS–polyacrylamide gels; a fluorograph of a representative experiment is shown in Figure 3. The entire 25% Cl_3CCOOH precipitates and 0.1 volume of the 10% Cl_3CCOOH precipitates are shown; the 2% Cl_3CCOOH precipitates, containing approximately equal quantities of histones as in the 10% Cl_3CCOOH precipitates (lanes a), are not shown. The ratios of HMG proteins 14 and 17 to core histones were found to be essentially the same among the three chromatin fractions, despite marked differences in the active sequence content of these fractions (Bloom & Anderson, 1978; Davie & Saunders, 1981; Kuehl et al., 1980). In order to confirm that the S1 fraction used in this study was also enriched in active sequences, the S1 DNA was hybridized to cDNA prepared against polyribosomal message. The S1 fraction, containing 5.5% of the total DNA in this experiment, hybridized to 35% of the cDNA, representing a 6.3-fold enrichment relative to total DNA.

The major chromatin fraction, S2, contained 70–80% of the DNA and correspondingly 70% of the HMG proteins (Figure 3) in accord with other reports (Davie & Saunders, 1981; Kuehl et al., 1980). Since the majority of the HMG proteins remained in the S2 chromatin, we sought to obtain further enrichment of HMG monomers by repeated micrococcal nuclease (MNase) digestion. Because soluble chromatin precipitates upon removal and readdition of divalent cations, potentially altering native nuclease accessibility, nuclei were maintained in magnesium buffer throughout the experiment by stopping each digestion with EGTA, and then calcium and MNase were restored for subsequent digestions (see Experimental Procedures). In this fashion, four successive sets of chromatin fractions were obtained.

Figure 4 shows the results of successive digestions assessed as nucleoprotein species in DNP gels (A) or for protein composition in SDS–polyacrylamide gels (B). Although the yield of S1 mononucleosomes dramatically diminished with each digestion step, the relative amount of monomer III in each S1 fraction was progressively enriched. The proteins in each S1 fraction (Figure 4B) were progressively enriched in HMGs 14 and 17, such that the final S1 contained an equimolar quantity of HMG 17 to core histones (Figure 4B, S1, lane 4). Each successive digestion had a drastically diminishing yield, however, and the final HMG-enriched fraction contained only 0.07% of the total DNA, obviating its extensive examination or a major role in transcriptional activity.

An interesting feature of this approach is that despite the progressive digestion of bulk chromatin to short oligomers and monomers most of the cleaved chromatin was not released as long as magnesium ion was present. Only upon addition of EDTA was the bulk chromatin solubilized, even at extensive digestion where monomers predominated (Figure 4A, S2). This effect is likely due to the effect of H1 in concert with divalent cations (Goodwin & Johns, 1978; Thoma et al., 1979; Zentgraf et al., 1980).

The assertion that HMG proteins bind in molar equivalence to nucleosomes associated with active sequences (Peters et al., 1979; Weisbrod, 1982; Levy et al., 1979; Weisbrod & Weintraub, 1979, 1981) has a corollary that tandem arrays of HMG monomers occur in active regions. Therefore, we investigated the active sequence enriched S1 fraction, obtained at very

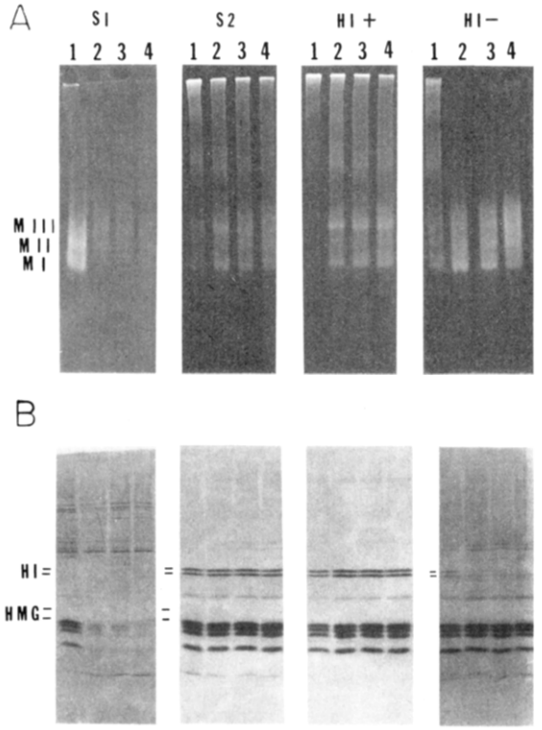

FIGURE 4: Sequential digestion of nuclei by MNase. Nuclei were digested to 2% acid solubility, the S1 was removed, and they were then resuspended and digested similarly 3 successive times. Fractions from each digestion were subjected to electrophoresis either in DNP gels and stained with ethidium (A) or in SDS gels and stained with Coomassie blue (B). Digestion is from left to right for each chromatin fraction indicated. The S2 fraction was adjusted to 0.1 M NaCl in order to separate the H1(−)-soluble chromatin from the H1(+)-precipitable chromatin, as noted in the figure.

limited digestions, for nucleosome oligomers as well as for HMG enrichment. We were never able to observe oligomers in the S1 fraction, even at very limited digestions (<1% acid-soluble DNA). Another approach to this question was to separate the S2 into H1(+) and H1(−) chromatins by precipitation of H1 chromatin with 0.1 M NaCl. The soluble H1(−) chromatin, like the S1, is usually monomeric also, but a sufficiently limited digestion, such as that shown in Figure 4, H1(−) lane 1, will yield multimers. However, we always found some H1 histone also to be present in nucleosome oligomers of this fraction, and the quantity of HMG proteins was not substantially enriched over that found in total chromatin. Due to the lack of enrichment of HMG proteins in these oligomers, and also to the absence of oligomers in the transcriptionally active S1 fraction, we are unable to support the contention that HMG monomers exist in tandem arrays. Since H1 and HMGs have different binding sites, however, it is still a possibility that HMG oligonucleosomes exist but contain H1 as well. Due to their occurrence primarily in mononucleosomes following all but the most brief digestion and their low abundance in those fractions, HMG nucleosomes appear to be interspersed with H1 monomers and other nucleosome species.

There is another feature of chromatin which may be emphasized here; although mononucleosomes containing one HMG protein migrate to the MII position, and those bound by two HMGs migrate to the monomer IIIB position, monomers bound by other non-histone proteins (NHP) can coelectrophorese to the same positions. Numerous types of less abundant NHPs bind nucleosomes, as shown in overexposures of two-dimensional gels (Albright et al., 1980; Annunziato et al., 1981), and are also evident by examination of the proteins

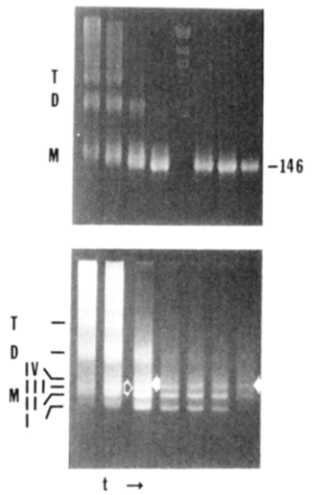

FIGURE 5: Continuous digestion of chromatin. Nuclei were digested with MNase in the presence of 100 mM KCl, samples were removed at increasing times, and the soluble chromatin was subjected to electrophoresis as DNA (top panel) or as DNP (bottom panel). The open arrow indicates the H1 monomer, MIIIA; the closed arrows indicate the HMG_2 monomer, MIIIB. The extents of digestion range from 2% acid solubility (first lane) to 20% (last lane).

in H1(−) mononucleosomes (Figure 4). In contrast, the H1(+) chromatin appears relatively deficient in NHP (Figure 4).

In a somewhat different approach, we found that the HMG monomers could be progressively enriched by extensive MNase digestion (Figure 5). Due to the solubility of these particles, they persist in solution to late stages of digestion, when H1 monomers and core particles have precipitated. The size of monomeric DNA in the early digestion stages ranged from 160 to 180 base pairs (bp), and the full range of mononucleosome complexity was observed (Albright et al., 1980; Annunziato et al., 1981). With progressive digestion, the DNA was trimmed to core size (146 bp), and the H1 and core particles precipitated, leaving the HMG monomers, MII and MIIB, in solution. The MNase resistance of these monomers (MII and MIIIB) was surprising in view of the reported DNase I sensitivity (Weisbrod & Weintraub, 1979, 1981; Weisbrod et al., 1980; Gazit et al., 1980). While this paper was in preparation, a report appeared (Kootstra, 1982) that corroborates the solubility and nuclease resistance of HMG nucleosomes and further showed that an apparently different class of particles is selectively sensitive to DNse I. The persistence of MII and MIIIB upon trimming of DNA by MNase to core length and concomitant loss of H1 (Noll & Kornberg, 1977) supports the evidence that the HMG binding sites are located differently from the H1 binding site (Albright et al., 1980; Mardian et al., 1980).

Reconstitution of HMG Mononucleosomes. We next sought to determine whether HMG proteins selectively bind nucleosomes of active chromatin, by reconstitution experiments. In initial experiments, we took the approach of isolating individual nucleoprotein monomer bands and then determining whether they would reconstitute faithfully to their original electrophoretic positions or would randomize among all monomer types upon reconstitution. Cells were uniformly labeled with [^{3}H]thymidine, chromatin was prepared by MNase digestion and subjected to electrophoresis in DNP gels, and the individual monomer bands, MI, MII, and MIII, were excised and electroeluted. There was a pronounced tendency for isolated monomers II and III to dissociate into progressively simpler species (Figure 6A, lanes c–e). Thus, MII gave rise to MI (Figure 6A, lane d), and MIII gave rise to MII and MI (Figure 6A, lane e).

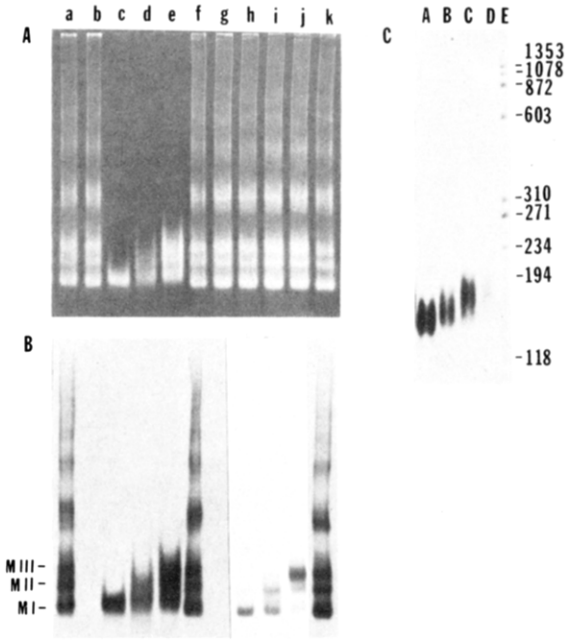

FIGURE 6: Fidelity of reconstitution of nucleosome monomer subtypes. [^{3}H]Thymidine-labeled chromatin was prepared and subjected to electrophoresis in DNP gels; the three main monomer types were located by UV shadowing, excised, and electroeluted. The purified monomers were then either subjected to electrophoresis directly or reconstituted in the presence of a 10-fold excess of unlabeled carrier chromatin. (Lanes a and k) Total soluble ^{3}H-chromatin; (lanes b and g) total soluble unlabeled chromatin; (lane c) MI; (lane d) MII; (lane e) MIII; (lane f) labeled chromatin reconstitute; (lanes h–j) reconstitutes with unlabeled chromatin: (h) MI, (i) MII, (j) MIII. (Panel A) Ethidium stain; (panel B) fluorograph. (Panel C) DNA size analysis of MI (A), MII (B), MIII (C), MIV (D), and marker fragments (E).

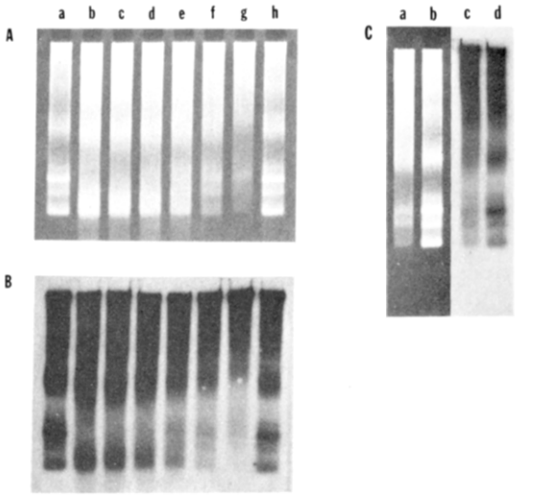

FIGURE 7: Reconstitution of salt-stripped chromatin with 0.35 M NaCl extracted nuclear proteins. The S1 and S2 fractions were pooled and washed with 0.45 M NaCl. (Lanes a and h) Unstripped total chromatin; (lane b) stripped chromatin; (lanes c–g) stripped chromatin reconstituted in progressive increments of 0.35 M NaCl extracted nuclear proteins. The DNP gel was stained (A) and then blotted to nitrocellulose and hybridized to [^{32}P]cDNA prepared against polyribosomal poly(A+) mRNA (B). (Panel C) Reconstitution of stripped nucleosomes with a 0.45 M NaCl extract of chromatin: (lanes a and b) ethidium stain; (lanes b and c) blot-hybridized cDNA autoradiogram; (lanes a and c) reconstitutes; (lanes b and d) control chromatin.

When each of the three monomer types was mixed with a 10-fold excess of unlabeled total chromatin as a donor for NHP (Figure 6A, lanes b and g), adjusted to 0.6 M NaCl, and reconstituted by step dialysis (Nelson et al., 1979), there was a strong tendency for monomer III, and to a lesser extent for monomer II, to reconstitute to its original position in spite of the existence of two HMG and one H1 binding sites on each of the three monomer species (Figure 6B, lanes h–j). The monomer I, under these conditions, reconstituted primarily to the monomer I position.

Upon measurement of the DNA size of each monomer type (Figure 6C), it was found that the MI contained an average size of 156 bp, MII 166 bp, and MIII 180 bp. Further, both isolated MIIIA (H1 monomers) and MIIIB (HMG monomers) reconstituted with the same kinetics, using a 0.35 M NaCl nuclear extract as the source of NHP (not shown); therefore, the type of accessory protein originally bound did not predetermine the reconstitution behavior of the isolated particle. We conclude, in agreement with Albright et al. (1980), that the ability of nucleosomes to reconstitute with HMG proteins is a strong function of DNA length; i.e., HMG reconstitution is preferential for chromatosomes (165 bp) over core particles (146 bp). However, as shown below, stripped monomers can be driven to any electrophoretic position by reconstitution with the appropriate quantity of salt-extracted nuclear proteins [see also Sandeen et al. (1980)].

Sequence Distribution among Mononucleosome Species. The sequence content of HMG proteins and other monomer subtypes was studied by reconstitution of salt-stripped chromatin with progressive amounts of 0.35 M NaCl extracted nuclear proteins, coupled with blot hybridization analysis of the monomer species. We reasoned that if HMG proteins preferentially bind active nucleosomes (Weisbrod & Weintraub, 1979, 1981; Weisbrod et al., 1980; Sandeen et al., 1980; Gazit et al., 1980), then transcribed sequences should preferentially appear in the HMG-containing monomer bands during titration, relative to the distribution of bulk DNA among various monomer types.

In attempts to perform reconstitutions with purified HMG proteins 14 and 17, the yield of purified proteins was low (note their abundance, Figure 2), the purity was variable, and the reconstitutions were problematic. Since there is no assay for the native state of HMGs, and purification involves the use of harsh denaturants, we chose to use the unfractionated 0.35 M NaCl nuclear extract as a source of native HMG proteins. In several reports (Weisbrod & Weintraub, 1979; Weisbrod et al., 1980; Gazit et al., 1980) and in our experience (e.g., see Figure 6), salt-dissociated nuclear proteins give highly reproducible reconstitution results.

HeLa cell chromatin was prepared by MNase digestion to 5% acid solubility, and the NHP proteins, including H1, were removed by washing with 0.45 M NaCl (Albright et al., 1980; Seale, 1981ab). The stripped chromatin was then reconstituted with increments of 0.35 M NaCl soluble nuclear proteins, subjected to electrophoresis in DNP gels, blotted on nitrocellulose, and hybridized to cDNA prepared against HeLa poly(A+) mRNA (Figure 7). The ethidium-stained pattern shows the progressive reconstitution of the stripped core into MII and MIII, and in sufficient NHP excess (lane g), most of the monomer is converted to MIII, with about 30% MII. Note the relative abundance of monomer species in the starting material (Figure 7A, lanes a and h). The hybridization pattern (Figure 7B) closely follows the staining pattern (Figure 7A). Controls have demonstrated that chromatin proteins do not adhere to the filter under these conditions and thus cause artifactual probe binding. There was no preferential mobilization of active sequences to the HMG bands (Figure 7A,B, lanes b–g), and no enrichment of active sequences in mononucleosome II or IIIB of native chromatin, over that of the bulk ethidium stain (Figure 7A,B, lanes a and h).

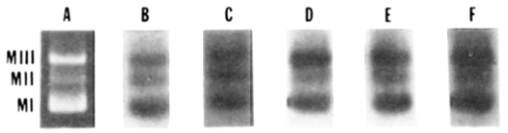

FIGURE 8: Blot hybridization analysis of Friend cell chromatin. The monomer region only is shown. (Panel A) Ethidium stain; (panels B and D) uninduced cells; (panels C and E) induced cells; (panels B and C) hybridized to β-major globin cDNA probe; (panels D and E) hybridized to fetal globin cDNA probe; (panel F) hybridized to inactive κ-chain cDNA probe.

The resolution of monomers reconstituted with the 0.35 M NaCl extract was always inferior to that of dissociation and reconstitution of unstripped chromatin (Figure 6). This is attributable, in part, to the lack of H1 in the 0.35 M extracts. When 0.45 M NaCl extracts were employed, the resolution of reconstituted monomers was improved. The kinetics of reconstitution of active sequences were still the same as those for the bulk profile (Figure 7C). Because the kinetics were the same, a single intermediate step in reconstitution is shown, demonstrating the lack of preferential mobilization of active sequences to the HMG bands.

The claim that active gene chromatin is selectively, and stoichiometrically, complexed with HMG proteins (Weisbrod et al., 1980; Weisbrod & Weintraub, 1981; Weisbrod, 1982) has a corollary that inactive chromatin is devoid of these proteins. We examined these complementary questions by measuring the abundance of both active and inactive single-copy gene probes among mononucleosome species. For this purpose, we used murine erythroleukemia cells for which appropriate cloned probes are available.

Nuclei were purified and digested under low ionic strength conditions; the soluble chromatin was subjected to electrophoresis in DNP gels, blotted to nitrocellulose, and hybridized. One hybridization compared the β-globin gene distribution among mononucleosomes from control or from hexamethylene bis(acetamide) (HMBA) induced cells which are stimulated 30-fold in globin mRNA synthesis (Nudel et al., 1977). Figure 8B,C shows that the β-globin gene has identical mononucleosome hybridization patterns in both differentiation states, and each has a distribution essentially identical with the bulk ethidium pattern (Figure 8A). Approximately half of the total hybridizing monomer was in the core. We showed in Figure 5 that digestion largely spares HMG-containing monomers; therefore, cores are derived primarily from digestion of other mononucleosome types (Todd & Garrard, 1979).

The embryonic globin gene, ϵY3, is not expressed in either control or HMBA-induced cells. When hybridization was performed with a cDNA probe to this gene, the monomer pattern in both induced and uninduced cells was also the same as that of bulk chromatin. In addition to this inactive gene, we have also used a probe for an inactive immunoglobulin light chain gene (lane F), with the same result.

The lack of enrichment for active sequences in HMG monomers is in concurrence with reports by Levinger et al. (1981) that there is little difference among hybridization patterns of electrophoretically separated nucleosomes probed with actively transcribed dihydrofolate reductase gene or with nontranscribed satellite DNA. These results are also in accord with evidence for the presence of HMG proteins in nontranscribed chromatin (Davie & Saunders, 1981; Kuehl et al., 1980).

Discussion

In this study, we have examined the abundance of HMG proteins in relation to that of active sequences in total chromatin, and in a chromatin fraction enriched in active sequences. The total amount of HMG proteins 14 and 17 in HeLa cells was found to be 1–2% of the mass of core histones, compared with values of 3% in calf thymus (Goodwin et al., 1973) and duck erythrocytes (Vidali et al., 1977), 4% in mouse P815 cells (Gabrielli et al., 1981), and 2% in trout testis (Kuehl et al., 1980). Conceivably, the abundance of HMG proteins could account for that required for the transcriptional activation of nucleosomes. At a ratio of 1 HMG protein to 8 core histones, the fraction of nucleosomes bound correlates with the fraction of active genes in chromatin. However, approximately half of the HMG nucleosomes bear two HMG proteins, diminishing the number of HMG monomers by 25%.

In a chromatin fraction that was 6-fold enriched for active sequences in accord with the report of Bloom & Anderson (1978), we found little enrichment of HMG proteins. Others have reported enrichment of HMG 14, but not HMG 17, in a transcriptionally active fraction (Gabrielli et al., 1981); note that the abundance of HMG 14 in mouse cells is 0.4% that of core histones, obviating stoichiometric equivalence in active sequences. Using nick-translation labeling of active sequences, Davie & Saunders (1981) found a 4-fold enrichment of labeled chromatin in fractions enriched 2-fold in HMGs 14 and 17. These authors, as well as others, stress the presence of HMGs 14 and 17 in nontranscribed regions (Barsoum et al., 1982; Davie & Saunders, 1981; Kuehl et al., 1980; Levinger et al., 1981).

We were unable to find evidence for HMG nucleosomes present in tandem arrays. This was based on the solubility of non-H1 nucleosomes. The S1 fraction, which is devoid of H1, is composed exclusively of monomers regardless of the extent of digestion. The H1(−) fraction, obtained by precipitation of H1(+) nucleosomes from the major soluble chromatin fraction, was primarily monomeric. At very low extents of digestion, a soluble H1(−) fraction could be obtained that was oligomeric, but the HMG content was not enriched, and a small amount of H1 was inevitably present on the soluble oligomers.

When we examined the distribution of both active and inactive sequences among native mononucleosome species by blot hybridization, there was neither a notable enrichment of active sequences nor a depletion of inactive sequences in HMG-containing monomers. Two recent reports are in agreement with these observations. Barsoum et al. (1982) found no enrichment of actively transcribed dihydrofolate reductase genes in HMG mononucleosomes of mouse cells, and Mathew et al. (1981) found no enrichment of globin sequences in HMG nucleosomes of avian erythrocyte chromatin. These observations are in conflict with the reports of transcriptional activation of nucleosomes by the binding of HMG proteins 14 and 17 to nucleosomes in stoichiometric equivalence (Weisbrod & Weintraub, 1979, 1981; Weisbrod et al., 1980; Weisbrod, 1982).

In reconstitution experiments of stripped nucleosomes with salt-extracted nuclear proteins, we found no evidence for enhanced affinity of active sequence nucleosomes for HMG proteins. Recently, Reeves & Chang (1983) reported that reconstitution of nucleosomes with HMGs 14 and 17 did not restore preferential DNase I sensitivity to active nucleosomes. In a titration of avian erythrocyte nucleosomes with purified HMG proteins, Sandeen et al. (1980) found a slight enrichment of globin sequences in the HMG-bound monomers; however, the DNA length of the reconstituted monomers was not determined. This is an important consideration in light of the evidence that, although HMG nucleosomes are stable

if the DNA is trimmed to core length, in reconstitution conditions the HMGs show a strong preference for chromatosomes over trimmed cores (Albright et al., 1980). The HMG column-bound, active nucleosome fraction reported by Weisbrod (1982) had chromatosome-length DNA on average, as compared to the nonretained nucleosomes which contained core-length DNA. Thus, preferential reconstitution may have resulted from DNA size rather than from an intrinsic transcriptional conformation of the receptor nucleosomes. The selectivity of HMG proteins for nucleosomes according to DNA length, rather than sequence content, has recently been corroborated by Swerdlow & Varshavsky (1983).

Other studies on HMG binding to nucleosomes indicate that the effect of binding is not to extend or loosen chromatin (McGhee et al., 1982) but to stabilize it (Harrington et al., 1982). HMG proteins confer DNase I resistance to bulk nucleosomes (Sandeen et al., 1980); in the present report, we found that native HMG monomers were also resistant to MNase in accord with the report of Kootstra (1982). Thermal denaturation studies indicate that the DNA exit points on the particle are stabilized by HMGs and that polynucleosomal chromatin assumes a more condensed, rigid conformation due to HMG binding (Sandeen et al., 1980; Sasi et al., 1982).

The properties of HMG proteins are difficult to reconcile with a primary role in transcriptionally active, DNase I sensitive chromatin. As a minimum value for transcriptionally active, hence DNase I sensitive chromatin, we will use the generally accepted figure of 10–20% of DNA as transcriptionally active. The total amount of DNase I sensitive chromatin is probably much larger for several reasons. First, rarely transcribed genes and rapidly processed regions of primary transcripts would contribute disproportionately small amounts of hybridizable material that are scored in quantitative hybridizations, relative to that of abundant and stable transcripts. Second, nontranscribed genes that were once active in ontogeny retain their DNase I sensitivity, while contributing no transcripts used for these estimates (Weintraub & Groudine, 1976; Wood & Felsenfeld, 1982; David et al., 1980). Third, extensive nontranscribed DNA regions are DNase I sensitive; for instance, the ovalbumin gene resides in a DNase I sensitive 100 kilobase pair domain, of which coding sequences comprise less than 20% (Lawson et al., 1982). A similar situation appears to exist for the globin gene in avian erythrocytes; although the boundaries of nuclease sensitivity have not been determined, the active β-globin gene and inactive (but DNase I sensitive) embryonic β-globin gene both reside within a larger DNase I sensitive domain (Wood & Felsenfeld, 1982). Thus, whatever the total percentage of DNase I sensitive chromatin is, it clearly is significantly greater than the amount of transcribed DNA and is not strictly related to active coding sequences. The kinetics of digestion of chromatin to acid solubility with DNase I are strikingly biphasic and may reflect the amounts of these sensitive and resistant chromatin regions. It is apparent either that HMG proteins are not responsible for the DNase I sensitivity of active sequences or that HMG proteins also reside outside them. The evidence presented in this report indicates that HMG proteins exist both in transcriptionally active and in nontranscribed chromatin.

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Investigation of the Structure, Protonation, and Reactivity of Tetraammine(imidodiphosphato)cobalt(III), a Substrate for Potato Apyrase†

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ABSTRACT: The present study was undertaken to evaluate the suitability of polyphosphoroamidates as probes of proton transfer steps in enzyme-catalyzed phosphoryl transfer reactions. An inert coordination complex, P^1,P^2-bidentate $Co(NH_3)_4PNP$, was prepared and crystallized in both its neutral and fully protonated (hydrochloride) forms for X-ray analysis. Crystals of the neutral form $[Co(NH_3)_4HP_2O_6NH{\cdot}3H_2O]$ are monoclinic with space group $P2_1/c$ ($Z = 4$) and cell dimensions $a = 8.582$ (6) Å, $b = 16.128$ (6) Å, $c = 10.730$ (4) Å, and $\beta = 126.23$ (5)°. The hydrochloride $[Co(NH_3)_4H_3P_2O_6NH^{2+}{\cdot}2Cl^-]$ crystallizes in the orthorhombic space group $P2_12_12_1$ ($Z = 4$) with cell dimensions $a = 7.088$ (2) Å, $b = 10.345$ (2) Å, and $c = 17.589$ (3) Å. Both structures were solved by the heavy atom technique and refined to R indexes of 0.055 and 0.057 for the neutral and hydrochloride forms, respectively. The six-membered chelate ring in the neutral form assumes the chair conformation while the hydrochloride is found in a conformation intermediate between the boat and twist-boat conformations. Addition of HCl to the neutral complex fails to protonate the imido nitrogen atom to the quaternary charged state, rather the phosphate oxygen atoms of the acidified complex accept the two protons donated by the chloride ions. This accounts for the observed stability of $Co(NH_3)_4PNP$ in 6 M HCl. The potato apyrase reaction was examined with $Co(NH_3)_4PNP$, $Co(NH_3)_4PP$, imidodiphosphate (PNP), and pyrophosphate (PP) as substrates. Both PP and PNP were hydrolyzed to orthophosphate, the $Co(NH_3)_4PP$ complex was hydrolyzed to $Co(NH_3)_4(P)_2$, and $Co(NH_3)_4PNP$ was converted to $Co(NH_3)_4PP$ via a $Co(NH_3)_4(PO_4)(PO_3NH_3)$ intermediate. The observations suggest that apyrase transfers a proton to the nitrogen atom of both PNP and $Co(NH_3)_4PNP$ during the course of catalysis.

Structural analogues of ATP that are resistant to enzyme action can be of great utility in the study of catalytic mechanisms for ATP-dependent enzymes. In one such analogue, adenyl-5′-yl imidodiphosphate (AMP-PNP),[1] the β,γ-bridge oxygen atom of ATP is substituted with a nitrogen atom. AMP-PNP is principally used to study ATP binding to enzymes which are unable to cleave the P–N linkage (Yount, 1975). Solution studies of imidodiphosphate (PNP) itself have shown that hydrolytic cleavage of the P–N bond does not occur readily unless the nitrogen atom is fully protonated (Quimby et al., 1960). Thus, it appears that only those enzymes which can transfer a proton to the imido nitrogen atom of AMP-PNP during catalysis should be capable of catalyzing γ-phosphoryl transfer from AMP-PNP while those enzymes which either do not normally protonate the β,γ-bridge oxygen atom of ATP or are unable to protonate the bridge nitrogen atom of

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; AMP-PNP, adenyl-5′-yl imidodiphosphate; PNP, imidodiphosphate; PN, phosphoroamidate; PP, pyrophosphate; P, orthophosphate; Pipes, piperazine-N,N'-bis(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

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