Partial inhibition of histone deacetylase in active chromatin by HMG 14 and HMG 17

R.Reeves[†] and E.P.M.Candido[†][†]Biochemistry/Genetics Program, Washington State University, Pullman, WA 99164, USA, and[†]Department of Biochemistry, University of British Columbia, Vancouver, B.C., Canada V6T 1W5

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ABSTRACT

Digestion of isolated Friend erythroleukemic cell nuclei with DNase I under conditions which selectively destroy the DNA of transcriptionally "active" genes releases into the supernatant fraction proteins of the non-histone "High Mobility Group" (HMGs). Two of these, HMG-14 and HMG-17 (identified by solubility in trichloroacetic acid, electrophoretic mobility on SDS-polyacrylamide gels and by amino acid composition) will partially inhibit the endogenous mouse cell histone deacetylase enzymes when added to in vitro assay mixtures. Other closely related proteins do not share this inhibitory ability and thus the reaction with the enzymes appears to be specific. Since these two HMG proteins appear to be preferentially associated with the "active" fraction of chromatin, these findings have important implications for possible models of eukaryotic gene regulatory mechanisms.

INTRODUCTION

Mononucleosomes released from isolated nuclei (or chromatin) by different nuclease digestion procedures appear to be very heterogeneous with respect to their content of H-1 histones, their content of associated non-histone chromatin proteins and in their levels of either variant or post-synthetically modified nucleosomal "core" histones (1-8). It is also becoming apparent that some of this inherent nucleosomal variability may have important functional significance in eukaryotic cells. For example, various nuclease digestion conditions that are capable of fractionating chromatin into transcriptionally active and inactive components (9-11) also result in the selective release into the "active fraction" of nucleosomes containing histones that are very highly acetylated relative to the levels of acetylation found on the histones in the inactive nucleosome fraction (12,13). These findings are in agreement with the original suggestion (14) that histone acetylation might play an important facilitating role in the activation and maintenance of chromatin for gene transcriptional activity (15,16) and this hypothesis has been given further support by recent findings from several laboratories

(17-19).

Non-histone chromatin proteins of the "high mobility group" (HMG) (20) are also found associated with mononucleosomes (4-6,21,22). Moreover, these proteins are likewise distributed in a distinctly non-random fashion among the chromatin subunits (4-6). It appears, for example, that the four main HMG proteins (HMG's 1, 2, 14, and 17) found in calf thymus and other mammalian cell types (21,23) may be preferentially associated with the regions of transcriptionally "active" chromatin recognized by short-term nuclease digestions (4-6, 22-24). Thus, DNase I digestion of chromatin under conditions which are known to selectively digest active genes, preferentially removes HMG proteins (23-25), while the selective digestion of globin gene sequences in chick erythrocyte chromatin by DNase I is abolished if these HMG proteins are first extracted from the chromatin preparations by 0.35 M NaCl elution (25,26). Even more importantly, it has been convincingly demonstrated (25,26) that reconstitution of the salt-depleted erythrocyte chromatin with either the entire 0.35 M NaCl fraction or a subclass from this fraction greatly enriched in only two of the HMG proteins, HMG 14 and HMG 17, results in the successful reconstitution of DNase I sensitivity of the globin genes. These results clearly demonstrate the importance of HMG 14 and/or HMG 17 proteins in helping to establish the "active" chromatin structure recognized by DNase I digestions in avian cells. However these, or homologous, HMG proteins may have similar functions in other types of eukaryotic cells as well. In recent work with the trout testis system, Levy and her colleagues have demonstrated that the trout-specific protein H6, which has many similarities to calf-thymus-HMG's 14 and 17 (27), is selectively solubilized and released when nuclei (or chromatin) are digested with DNase I (28,29). Furthermore, H6 has also been identified as a stoichiometric component of a distinct subset of trout testis nucleosomes derived from transcriptionally active genes by limited digestion with micrococcal nuclease (30,31).

Since both HMG proteins and hyperacetylated histones seem to be preferentially associated with nucleosomes derived from transcriptionally active genes, it is logical to question whether these two features of specialized chromatin are in any way related. We have previously reported (32) that digestion of nuclei from Friend erythroleukemic cells with DNase I releases into the supernatant fraction an inhibitor of the histone deacetylase enzymes (presumably derived from the transcriptionally active chromatin). In this communication we demonstrate that among the proteins released by DNase I

digestion of Friend cell nuclei are HMG's 14 and 17. Furthermore, we show that in vitro these two HMG proteins, either together or separately, can act as inhibitors of the histone deacetylase enzymes.

MATERIALS AND METHODS

Cell Line and Culture Conditions:

Friend erythroleukemic cells, clone 745A, were maintained and passaged using the techniques described in (33). The culture medium contained 90% Dulbecco's Modified Eagle's Medium, 10% fetal calf serum and 100 $\mu\text{g/ml}$ each of sodium penicillin G and dihydrostreptomycin. ^3H -acetate-labeled histones for use in the deacetylase enzyme assays (described below) were isolated by described methods (33) from Friend cells labeled in vivo with ^3H -acetate (2×10^9 cells were incubated for 2 hr with 150 $\mu\text{Ci/ml}$ of ^3H -acetate, specific activity $>500 \text{ mCi/mmole}$, New England Nuclear) in the presence of 5 mM sodium butyrate to inhibit the deacetylase enzymes (33).

DNase I Digestion of "Active" Chromatin:

Nuclei were isolated from frozen cells by previously described procedures (33) and digested with pancreatic deoxyribonuclease I (E.C. 3.1.4.5, Sigma) under conditions known to preferentially destroy the DNA in "active" chromatin (34). Nuclease digestions were continued until about 12-15% of the chromatin DNA was released as acid-soluble (7% perchloric acid, 4°C) nucleotides and small fragments. Nucleic acid hybridization experiments (not shown) confirmed that the transcriptionally active genes were preferentially destroyed under these digestion conditions (25 units/ml of DNase I, 4 mg/ml of chromatin, 4 min at 37°C). The proteins released into the supernatant by DNase I digestion were fractionated by differential acid precipitations (35) to reveal the presence of the HMG proteins in this fraction (see below).

Isolation of the High Mobility Group (HMG) Proteins:

HMG proteins were prepared from isolated Friend cell nuclei by a modification of the method of Goodwin et al. (35). Nuclei were washed several times with TMK buffer [10 mM Tris (pH 8.0), 1 mM MgCl_2 , 25 mM KCl and 1 mM phenylmethylsulfonylfluoride (PMSF)] and then lysed by homogenization in a solution containing 10 mM Tris (pH 7.4), 0.2 mM EDTA and 1 mM PMSF. The resulting chromatin was recovered by centrifugation through a sucrose cushion (33). The chromatin pellet was extracted three times with 0.35 M NaCl/10 mM Tris (pH 7.4)/1 mM PMSF, by homogenization with a loose-fitting Dounce homogenizer and centrifuged at $8000 \times g$ for 10 minutes to yield a 0.35 M NaCl

eluate. The eluate was spun at 27,000 x g for 30 minutes. Proteins were then separated on the basis of their solubility in trichloroacetic acid (TCA) (35), washed in acetone/HCl (0.1 N HCl:acetone, 3:1 v/v), followed by acetone, and assayed on 15% or 18% SDS polyacrylamide gels by the electrophoresis procedure of Laemmli (36). Fractions were obtained as follows: 1) the low mobility group (LMG) proteins were removed from the 0.35 M NaCl extract by precipitation with 2% TCA; 2) HMG proteins 1 and 2, and a number of other non-histone proteins that remained soluble in 2% TCA were selectively removed by raising the acid concentration of the eluate to 10% TCA; 3) only HMG proteins 14 and 17 (and a minor contaminant of higher molecular weight) remained soluble in 10% TCA and these were recovered by precipitation after raising the acid concentration to 20%. Pure HMG 14 and 17 proteins were isolated individually by elution from an 18% SDS polyacrylamide gel. The gel slices were finely minced and heated to 65°C for 2 hours in 10 mM Tris (pH 7.4)/0.1% SDS. The gel pieces were centrifuged out, the supernatant was made 0.1 N in HCl, 3 volumes of acetone was added, and the mixture placed at -70°C for 1 hour. Precipitated SDS was spun out in an Eppendorf microfuge, and the HMG proteins were then precipitated by adding an additional 3 volumes of acetone.

Spectrophotometric quantitation of the purified HMG proteins was by the Coomassie Blue G-250 protein assay procedure of Bradford (37) using either purified HMG proteins or purified H-1 histones as reference standards.

Amino Acid Analysis:

Analysis of the combined HMG 14 and 17 proteins isolated from Friend cells was performed on an automatic amino acid analyzer using a single column system, after hydrolysis of the samples in 6 N HCl at 110°C for 24 or 48 hours in evacuated sealed tubes. No corrections were made for hydrolytic losses. The amino acid composition of the mouse HMG's was compared to the published values for purified HMG 17 (38) and HMG 14 (39) obtained from calf thymus.

In Vitro Histone Deacetylase Enzyme Assays:

Histone deacetylase enzyme assays were performed essentially as described previously (33) except that the reaction mixtures were terminated by boiling prior to extraction of the released (^3H)-acetic acid from the mixtures with ethyl acetate. Briefly, suspension solutions of isolated nuclei (about 10^8 - 10^9 per ml) were divided into aliquots (200-300 μl) and each aliquot received an equal amount of ^3H -acetate-labeled histones (2.2×10^6 - 16×10^6 cpm/mg, obtained as described above). Various components, such as

HMG proteins, were then added to the reaction mixtures at the concentrations desired and the mixtures incubated at room temperature (22°C) for 4 hours. The reactions were terminated by boiling and 10 μ l of concentrated HCl was added to convert any acetate released in the mixture to the protonated form. The ^3H -acetic acid was then isolated from the mixtures by two extractions with 0.5 ml of ethyl acetate, and the ethyl acetate phases were counted in Aquasol scintillation fluid (New England Nuclear). Each deacetylase enzyme assay series contained a boiled reaction mixture (in which the enzyme activity was destroyed) to give the background values for the amount of ^3H -acetate released. For experiments involving endogenously labeled, (^3H)-acetate containing histones, about 10^9 log growth phase (control) Friend cells were re-suspended in fresh medium containing 5 mM sodium butyrate and 280 $\mu\text{Ci/ml}$ of (^3H)-acetate (Spec. Act. >500 mCi/mole; New England Nuclear) and grown for 12 hr at 37° with periodic adjustments of the medium pH when required. Nuclei from these in vivo labeled cells (containing hyperacetylated histones labeled with ^3H -acetate) were isolated as previously described (33) and used in the standard histone deacetylase enzyme assays described below except that no exogenously labeled histones were added to the reaction mixtures. Where required, isolated Friend cell HMG proteins (at the concentrations indicated in the text) were added to the reaction mixtures to test for enzyme inhibition. The various chemicals used and the additions to the enzyme assays noted in the text were obtained as follows: poly-L-lysine hydrobromide was obtained from Sigma; trypsin and trypsin inhibitor (soy bean) were from Sigma; purified H-1 histones and the trout-specific non-histone protein H6 were isolated from trout testis by published procedures (27).

RESULTS

Figure 1A shows that digestion of Friend cell nuclei with DNase I under conditions known to: 1) preferentially destroy the DNA of transcriptionally active, or potentially active, genes (34); and 2) release into the supernatant fraction a soluble inhibitor of the histone deacetylase enzymes (32), also releases from the chromatin a complex set of non-histone chromatin proteins. Furthermore, if the protein-containing supernatant is made 10% with respect to trichloroacetic acid (TCA) and the insoluble proteins removed by centrifugation, two main proteins, with mobilities on SDS polyacrylamide gels similar to those of the "high mobility proteins" HMG 14 and HMG 17, remain in the DNase I-derived supernatant.

Figure 1B shows that proteins with similar acid-solubility properties

Figure 1: SDS-Polyacrylamide Gels of DNase I-Released Chromosomal Proteins (Part A) and Trichloroacetic Acid Fractionated, 0.35 M NaCl Extracted, Nuclear Proteins (Part B). These slab gels contained 15% acrylamide except for those labeled "HMG 14 and 17" which contained 18% acrylamide. The soluble chromatin proteins were fractionated with TCA as described in the text. The molecular weight marker proteins and the marker histones were obtained from Sigma.

and electrophoretic mobilities to those released by DNase I digestion of nuclei can be obtained from the 0.35 M NaCl extracted proteins isolated from Friend cell nuclei by the method of Goodwin et al. (35). In this procedure, the 0.35 M NaCl extract is successively made 2% with respect to TCA concentration (to precipitate the low mobility group proteins), 10% in TCA (to remove the HMG 1 and HMG 2 proteins and other high molecular weight proteins) and finally 20% in TCA (to precipitate HMG 14 and HMG 17 which are soluble in 10% TCA).

Figure 2 shows the profiles on 15% polyacrylamide-SDS gels of the 10% TCA soluble proteins from Friend cell nuclei, compared to the HMG proteins from calf thymus nuclei. It is known that calf thymus HMG-14 and HMG-17

Figure 2: Comparison of the HMG proteins from calf thymus and Friend erythroleukemic cells. Lanes 1 and 8, molecular weight markers. Lanes 2 and 5, total proteins extracted from isolated nuclei with 0.35 M NaCl (including HMG's 1 + 2, ◆; HMG 14, ○; and HMG 17, ●). These proteins were a generous gift from Shirley Welch of Oregon State University; Lanes 3 and 7, marker calf thymus histones (Sigma); Lane 4, 10% Trichloroacetic acid (TCA)-soluble HMG proteins extracted from the nuclei of Friend erythroleukemic cells exposed for 24 hr to 5 mM sodium butyrate prior to extraction (these cells seem to contain primarily the HMG-17 protein, ▲); Lane 6, 10% TCA-soluble HMG proteins isolated from the nuclei of control (non-butyrate treated) Friend erythroleukemic cells. These cells seem to contain both HMG-14 (◆) and HMG-17 (●) proteins. These gels were stained with Coomassie Brilliant Blue R (Sigma) which stains all of the HMG-14 and HMG-17 proteins purple and the rest of the proteins on the gel blue (40).

migrate anomalously on SDS gels due to their interactions with the gel matrix (35,40). Thus, calf thymus HMG-17, with a known molecular weight of 9,247 daltons, has about the same relative mobility as histone H3, which is approximately twice as large. Similarly, calf thymus HMG-14 migrates just behind HMG-17, and has a molecular weight of about 10,390 daltons.

It is seen in Figure 2 that HMG-14 and HMG-17 from Friend cells migrate more slowly than H3, and have apparent molecular weights slightly larger than their calf thymus counterparts. For example, the putative Friend cell HMG-17 has an electrophoretic mobility similar to that of HMG 14 from calf, whereas the Friend cell HMG-14 migrates more slowly still and does not co-migrate with any of the major calf thymus HMG proteins.

Another finding illustrated in Figure 2 is that of an unexpected effect of sodium butyrate treatment on the HMG proteins of Friend erythroleukemic cells. Treatment of Friend cells for 24 hours with 5 mM sodium butyrate induces hyperacetylation of the nucleosomal core histones by inhibiting the histone deacetylase enzymes (33) and, during the same time period, seems to induce new genomic expression in these cells (19). Figure 2, lane 4, shows that following butyrate treatment the Friend cells seem to contain primarily HMG-17 in the 10% TCA-soluble protein fraction of non-histone chromatin proteins. Little, if any, HMG-14 is present in these fatty acid treated cells. This result contrasts markedly with the situation found in control, non-treated, Friend cells where the 10% TCA-soluble protein fraction usually contains about equal amounts of HMG-14 and HMG-17 (Figure 1), or, occasionally, slightly more HMG-14 than HMG-17 (Figure 2, lane 6). The possible reasons for this butyrate-induced shift in the relative concentrations of the HMG proteins in Friend cells are currently being investigated. Regardless of the causes, this phenomenon facilitates the isolation of HMG-17 in preparative quantities.

We have also carried out amino acid analyses of the 10% TCA soluble proteins shown in Figure 1B, and the results are summarized in Table 1. In this table the amino acid composition of the combined HMG 14 and HMG 17 proteins of mouse (assumed to be in a 1:1 ratio) is compared to the known amino acid compositions of calf thymus HMG 14 (39) and HMG 17 (38) combined in a similar 1:1 mass ratio. From this table it is seen that all of these HMG proteins are very high in their concentrations of lysine, alanine, glutamic acid and aspartic acid and very low in their contents of histidine, phenylalanine, tyrosine, isoleucine, methionine and cysteine. Thus the amino acid compositions of the Friend cell HMG's 14 and 17 are similar to those of the corresponding calf thymus proteins. The discrepancies in amino acid composition between the proteins in mouse and calf (e.g., glycine) probably result from either a minor contamination of the mouse HMG protein sample with a protein of higher molecular weight (which is present on the gels shown in Figure 1 but doesn't reproduce well in the photograph) or from inherent variations between the proteins in these different species.

In summary, we believe that the Friend cell proteins identified as HMG's 14 and 17 in Figure 2 are probably homologous to those of calf thymus for the following reasons: 1) The acid-solubility properties of all of these proteins are similar; 2) The electrophoretic mobilities of the respective proteins from the two different sources on SDS gels are similar (but not identical) to

TABLE 1

Amino Acid Composition (mol %)⁺ of Isolated HMG
Proteins from Friend Erythroleukemia Cells
and from Calf Thymus*

Amino Acid	Friend Cell HMG 14 + HMG 17	Calf Thymus ^g HMG 14 + HMG 17 (1:1 ratio)	Calf Thymus HMG 14	Calf Thymus HMG 17
Asp	11.94	9.55	8.3	10.8
Thr	4.0	2.6	4.1	1.1
Ser	7.48	5.05	8.0	2.1
Glu	17.7	13.5	17.5	9.5
Pro	6.92	9.9	8.1	11.7
Gly	15.0	8.15	6.4	9.9
Ala	11.29	15.7	14.8	16.6
Cys	--	--	--	--
Val	5.32	2.9	4.0	1.8
Met	Trace	0.05	0.1	--
Ile	Trace	0.15	0.3	--
Leu	1.0	1.45	2.0	0.9
Tyr	Trace	0.1	0.2	--
Phe	Trace	0.15	0.3	--
His	--	0.1	0.2	--
Lys	17.04	21.5	21.1	21.9
Arg	3.23	4.55	5.4	3.7

⁺No corrections were made for hydrolytic losses.

*The amino acid composition of calf thymus HMG 14 is from (39) and of HMG 17 from (38).

^gSince the amino acid composition of the isolated Friend cell HMG proteins is from a mixture of HMG 14 and HMG 17 proteins in about a 1:1 mass ratio (Fig. 1), the calf thymus HMG's were also averaged in this column to give approximately comparable amino acid values.

each other when compared to the mobility of histones and other marker proteins separated on the same gels (Figure 2); 3) The amino acid composition of the Friend cell proteins is quite similar to that of calf thymus HMG's 14 and 17 (Table 1); and 4) As noted by others (40), Coomassie Blue stains calf thymus HMG-14 and HMG-17 differently from other proteins. Thus, these two proteins from calf thymus stain purple while the other HMG proteins stain blue, as do most cellular proteins. The Friend cell proteins labeled HMG-14 and HMG-17 in Figure 2 (Lanes 4 and 6) also stain purple while all other Friend cell proteins so far observed stain the usual blue. The biochemical reason for this differential color reaction is unknown but it seems to be a useful

criterion for distinguishing this group of HMG proteins from other non-histone chromatin proteins on gels.

We next investigated the possibility that the 10% TCA-soluble HMG proteins isolated from Friend cell chromatin might act as inhibitors of the histone deacetylase enzymes in our standard in vitro enzyme assay procedure (33). Figure 3 shows the results of these experiments with mixed populations of HMG's 14 and 17 added in increasing concentrations to the assay mixtures. It is evident from this figure that increasing concentrations of these HMG proteins do, indeed, inhibit the activity of the deacetylase enzymes. However, two other points should be made concerning these data: 1) there is considerable variability from one preparation of isolated nuclei to another in the amount of enzyme inhibition observed for a given batch of HMG proteins. (Each different symbol in Figure 3 represents a different nuclear preparation used in the enzyme assays.) For example, the HMG 14 and 17 proteins (at the highest concentrations used) gave a range of inhibition of the deacetylase

Figure 3: In vitro Inhibition of Nuclear Histone Deacetylase Enzymes by Exogenously Supplied 10% TCA-Soluble HMG Proteins (A Mixture of HMG 14 and HMG 17). Each symbol represents a separate enzyme assay series involving different isolated nuclear preparations and often different HMG preparations as well. See Materials and Methods for assay conditions.

enzymes of between 26% and 64% (with an average of 45% inhibition) with different preparations of nuclei (Figure 3). The reason for this variability is unknown but it should be emphasized that in no experiment did these HMG proteins fail to give noticeable inhibition of the enzymes; and 2) even at the highest HMG concentrations used (about $200 \mu\text{g}/10^9$ nuclei/ml) only about 50% (avg. = 45%) of the histone deacetylase enzyme activity was inhibited by these non-histone proteins.

That the inhibition of the deacetylase enzymes observed in Figure 3 was due to the HMG 14 and 17 proteins added to the reaction mixture and not to the minor protein contaminant also present in the 10% TCA soluble eluant (see above), is verified by the results shown in Figure 4. In this experiment electrophoretically purified HMG 14 (solid circles) or purified HMG 17 (open circles) proteins were added individually to the enzyme reaction mixtures. It is seen that either of these proteins, individually, will give about the same amount of deacetylase inhibition (within error) as the two HMG proteins mixed together. Also shown in this figure are the results obtained when a mixed population of HMG 14 and 17 proteins are digested with trypsin (and the trypsin subsequently inactivated with soy bean inhibitor) prior to being used in the enzyme assays (solid squares). It is evident that the inhibitory ac-

Figure 4: In vitro Inhibition of Nuclear Histone Deacetylase Enzymes by Exogenously Supplied, Individually Purified, HMG 14 and HMG 17 Proteins. O—O, HMG 17; ●—●, HMG 14; ■—■, control experiment in which a mixed population of HMG 14 and 17 was digested with trypsin prior to use in the enzyme assay.

tivity of the HMG proteins is destroyed by trypsin digestion. Another experiment demonstrating this point is shown in Table 2.

A number of additional control experiments were performed to investigate the specificity of the inhibition of the histone deacetylase enzymes by HMG's 14 and 17. The results of some of these controls are shown in Table 2. For example, it was found that the inhibitory activity of the HMG's was stable to boiling prior to their use in the enzyme assays. This could explain why these small proteins still retain their activity after the rather harsh treatment of exposure to high concentrations of acid (10-20% TCA). Additionally, it was found that other small, charged nuclear proteins such as histone H-1 and HMG proteins 1 and 2, as well as the trout testis non-histone protein H6 [which is similar to HMG's 14 and 17 (27)] do not cause noticeable amounts of inhibition of the deacetylase enzymes of Friend cells when added to the reaction mixtures. Furthermore, poly-L-lysine also lacks any inhibitory activity in these assay mixtures. On the contrary, a slight, non-specific stimulation of deacetylase activity was observed when HMG's 1 and 2, H1, H6 or polylysine were added to the nuclear preparations.

It should be noted here that the electrophoretic profiles of the total 0.35 M NaCl-extracted proteins isolated from Friend cell nuclei (Figure 1B), as well as the profiles of the subfraction of proteins in this mixture that are soluble in 2% TCA, are much more complex than the profiles of similar protein extracts obtained from calf thymus nuclei (c.f. Figure 2 and references 20 and 40). For example, as shown in Figure 1B, the 2% TCA-soluble protein extract contains many protein species, including proteins that co-migrate on these gels with marker histones. Therefore the results reported in Table 2 indicating that Friend cell proteins HMG-1 + HMG-2 (defined as those proteins which are soluble in 2% TCA and insoluble in 10% TCA) do not cause an inhibition of the histone deacetylase enzymes when added to an in vitro assay mixture means, in reality, that neither these HMG proteins nor the other proteins also present in this fraction inhibit these nuclear enzymes.

All of these results strongly suggest that the inhibition of histone deacetylases by HMG's 14 and/or 17 in Friend cells is a specific reaction.

In the above experiments, exogenous ^3H -acetate labeled histones were added as substrate for the deacetylase. Since the mode of binding of the added histone with the chromatin is unknown, it could be argued that the interaction between the histone deacetylase and its substrate is abnormal, and that the results may therefore not reflect the action of a physiolog-

TABLE 2
Control Experiments on Histone Deacetylase Activity^a

Protein	Approximate Protein Concentration ($\mu\text{g/ml}$) per Reaction ^b							Maximum % Inhibition
	None	Boiled (bkg) ^c	20	50	100	200	300	
HMG's 14+17	9,730	472	9,049	7,104	6,033	4,670	N.D.	52%
boiled HMG's 14+17	3,996	1,400	N.D.	3,877	2,962	2,179	N.D.	45.5%
trypsin- digested HMG's 14+17 ^d	3,996	1,400	N.D.	3,861	3,964	3,901	N.D.	3.38%
HMG's 1+2 ^e	11,171	824	N.D.	N.D.	13,045	13,086	N.D.	0%
H-1	11,171	824	N.D.	N.D.	13,044	12,650	N.D.	0%
poly- lysine	11,171	924	N.D.	13,000	13,717	13,167	N.D.	0%
trout testis H6	4,956	94	N.D.	5,568	5,975	6,027	5,767	0%

^a Each reaction mixture contained approximately 10^9 isolated nuclei per ml. Exogenous ^3H -acetate labeled histones were added to each reaction mixture (200–300 μl volume), along with the indicated concentrations of tested proteins. After 4 hours at room temperature, the amount of free ^3H -acetate (cpm) released from the added histones was determined as previously described (33) and this value is listed in the table.

N.D., not determined.

^b These protein concentration values are only approximate since the various experiments listed in the table were not all performed at the same time and the actual concentrations added varied slightly from experiment to experiment.

^c The nuclear reaction mixture was boiled prior to the addition of exogenous proteins, or ^3H -acetate labeled histones, to destroy endogenous enzyme activity.

^d HMG's 14+17 were digested for 45 min at 22° with 50 $\mu\text{g/ml}$ trypsin (Sigma, 3x crystallized) and soy bean trypsin inhibitor (Sigma) was then added to 100 $\mu\text{g/ml}$.

^e As explained in the text, this fraction contains those proteins initially extracted from Friend cell nuclei with 0.35 M NaCl and which are soluble in 2% TCA but insoluble in 10% TCA. As shown in Fig. 1 this fraction, in addition to HMG's 1 and 2, contains a large number of other proteins.

ically significant process. To test this possibility, we labeled Friend cells *in vivo* with ^3H -acetate, and used chromatin from such cells for assays of deacetylase activity directly, i.e. without exogenously added histone. In this situation, both the deacetylase and its histone substrate have been assembled into the chromatin *in vivo*, and hence should interact in a normal manner.

Figure 5 shows the results of such an experiment. It is seen that deacetylase activity is readily measured in these preparations (sample B); furthermore, the addition of HMG 14 + 17 clearly inhibits the enzyme (samples C, D and E), to a maximum of 46% in this case. Thus HMG's 14 + 17 can inhibit histone deacetylation in an endogenous chromatin preparation.

DISCUSSION

The results reported here demonstrate that the "high mobility group"

Figure 5: Histone deacetylase activity in nuclear preparations from cells labeled *in vivo* with ^3H -acetate. 10^9 cells were labeled for 12 hrs in medium containing 5 mM butyrate and 280 $\mu\text{Ci/ml}$ of ^3H -acetate. Nuclei were prepared, and incubated for 4 hrs at 22° . Released ^3H -acetate was extracted and counted as previously described (33).

A, nuclei heated at 100° for 2 min prior to assay.

B, deacetylase activity in control nuclei.

C,D,E, sample as in B, but containing 50, 100 and 200 μg respectively of 10% TCA soluble HMG proteins.

proteins 14 and 17, isolated from Friend erythroleukemic cells can, either individually or together, partially inhibit (up to about 50%) the histone deacetylase enzymes of these cells. This effect appears to be rather specific for these particular HMG proteins since a number of other similar proteins tested did not cause such inhibition. In particular, the trout testis non-histone protein H6, which is in many respects quite similar to calf thymus HMG's 14 and 17 (27) did not inhibit the mouse cell deacetylase enzymes, suggesting that there may be some species specificity involved in the proteins that regulate the deacetylases in vivo.

From Figures 3, 4 and 5, two notable points emerge. First, even at the highest concentrations of HMG's used, about 50% of the endogenous histone deacetylase remains active in the isolated nuclear preparations. There are a number of possible explanations for this finding but the two that come most immediately to mind are: 1) there may be other inhibitors of the deacetylases (besides the HMG's) present in these cells that are necessary to block the remaining resistant enzyme activities; and 2) there may be chromatin binding problems (steric or mass action) involved in the association and disassociation of the free HMG proteins when they are added to chromatin that already contains these proteins (22,26), and this might interfere with their inhibitory action. The second point to notice is that the effects of the individual HMG's 14 and 17 on the deacetylase enzymes are not additive. This suggests that the two proteins may be recognizing the same binding sites in chromatin.

The highest concentrations of HMG's 14 and 17 used in these studies (200 $\mu\text{g/ml}$ for 10^9 nuclei/ml) represents about one HMG molecule per nucleosome (assuming all of the HMG proteins bind to chromatin, which seems very unlikely). This concentration is about ten times higher than the one in 10 to one in 20 HMG's/nucleosome found by Weisbrod and Weintraub (25) to be necessary in reconstituting the DNase I sensitivity of the globin genes in chromatin that had been previously depleted of its HMG's by salt extraction. This high HMG concentration may have been necessary because exogenously supplied HMG's do not seem to bind readily to nucleosomes already containing these proteins (22,25,26) (which is the case with our chromatin preparations). However, other explanations are also possible. The amount of inhibition of the histone deacetylase enzymes observed with purified HMG proteins is in the same range as that observed with 1-5 mM concentrations of sodium butyrate, which also inhibits these enzymes in Friend erythroleukemic cells (33).

Our data are consistent with the idea that the deacetylases are probably

very active in the bulk of the chromatin (80-90%) which is transcriptionally inactive, and which probably contains primarily unacetylated histone species; these enzymes, we hypothesize, are less active (possibly as a result of their association with HMG 14 and/or 17) in transcriptionally active chromatin, which probably contains hyperacetylated histones (12,13). Thus, we speculate that the deacetylase inhibition that is observed when exogenous proteins are added to nuclei may result from the partial inhibition of the normally active enzymes found in the "inactive" chromatin.

A number of important questions are raised by the results reported here. For example, how do the HMG's bind to chromatin to inhibit the histone deacetylase enzymes? Do they bind to the histones (or nucleosomes) themselves in some way and thus block the access of the deacetylase enzymes to their substrates or do they interact directly with the enzymes to inhibit their action? The answers to these questions are not known at present but are amenable to experimental solutions.

In conclusion, the purified HMG proteins 14 and 17 in mouse cells partially inhibit the endogenous histone deacetylase enzymes in these cells, by a mode of action that remains to be elucidated. These findings have important implications for possible models of eukaryotic gene regulatory mechanisms.

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