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Effect of high mobility group nonhistone proteins HMG-20 (ubiquitin) and HMG-17 on histone deacetylase activity assayed in vitro

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ABSTRACT

We have used a method previously described by Reeves and Candido (1) to partially release histone deacetylase from cell nuclei together with putative regulators of the enzyme. Histone deacetylase released from testis cell nuclei and its putative regulators were separated by gel filtration on Sepharose 6B. A peak of low molecular weight contains a heat-stable factor that stimulates histone deacetylase in vitro. Many of the properties of the activator coincide with those of the protein HMG-20 (ubiquitin). Ubiquitin isolated from testis cell nuclei stimulated histone deacetylase in vitro. It has been suggested that HMG-17 partially inhibits histone deacetylase in Friend cell nuclei (2). In our system, HMG-17 shows no inhibitory effect on histone deacetylase activity.

INTRODUCTION

Histones can be modified by acetylation in two different ways. The NH_2 of the amino-terminal serine in histones H1 , H2A and H4 is permanently acetylated with no significant turnover of this group. In contrast, there is an extensive acetylation and deacetylation of lysine residues in the amino-terminal regions of all nucleosomal histones H2A, H2B, H3 and H4 (3).

Numerous correlations have been observed between acetylation of histones and gene activity (4). An increase of histone acetylation precedes the induction of new RNA synthesis in mitogen-stimulated lymphocytes (5), in regenerating liver (6) and in hormone stimulated target

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cells (7). Conversely, in the mature sperm of Arbacia lixula, where no RNA synthesis occurs, histones appear only in their nonacetylated forms (8). The transcriptionally active macronucleus of Tetrahymena pyriformis contains highly acetylated histones, whereas the histones of the micronucleus are not acetylated (9).

Histone acetylation may play a critical role in permiting DNA interactions with RNA polymerase and other nonhistone proteins. The acetylation of the amino-terminal arms of the histones may result in a relaxation of DNA twists within the nucleosome and also could have long-range effects on chromatin coiling. Such a process would offer a structural basis for permiting DNA interactions with chromosomal proteins involved in transcription.

At present, is uncertain whether acetylation of histones in active genes is metabolically stable or unstable (10). If acetylations are stable only at few specific chromatin sites but unstable at the nonspecific loci, histone deacetylase should be inhibited at the specific sites by regulatory molecules as has been proposed for HMG-14 and HMG-17 (2). If alternatively, in active genes the turnover of acetyl groups on the core histones of nucleosomes is extremely rapid (11) and acetylations are stable only at the nonspecific loci of chromatin, histone deacetylase should be active in transcriptionally active chromatin.

An increased rate of deacetylation may be ascribed to an increased accessibility of histone molecules to the deacetylase and/or to a higher enzymatic activity in transcribing chromatin. Assuming the second possibility, it seemed likely that deacetylase activators could be present in chromatin. To test the idea we have used a method previously described by Reeves and Candido (1) that partially

releases histone deacetylase from cell nuclei together with putative regulators of the enzyme. We report here the purification of a heat-stable activator of histone deacetylase with many properties in common with the protein ubiquitin. We also show that in our system the nonhistone chromosomal protein HMG-17, previously reported to inhibit histone deacetylase in Friend cell nuclei (2), shows no inhibitory effect on histone deacetylase.

MATERIALS AND METHODS

Nuclear isolation and nuclease digestion.

Nuclei were isolated from fresh rooster testis by the sucrose procedure previously described (12) except for the buffer used (0.01 M Tris-HCl, pH 7.9, 0.01 M NaCl, 10 mM $MgCl_2$ containing 0.25 per cent Triton X-100 and 1 mM PMSF). Purified nuclei were digested with DNase I (Sigma) under conditions known to preferentially destroy active genes(13). After 5 min. at 37°C about 10 per cent of the chromatin DNA was released. Digestion was terminated by chilling and immediately adding EDTA to a final 5 mM concentration.

Preparation of activator-deficient histone deacetylase.

The soluble material recovered by centrifugation at 12,000 x g for 5 min. after DNase I digestion of nuclei was made 0.35 M in NaCl and 20 ml were applied to a Sepharose 6B column, 2.5 x 100 cm(14). Fractions of 8 ml were collected. The eluate was monitored for histone deacetylase activity and histone deacetylase activator as described below.

Preparation of histone deacetylase activator.

Fractions 57 to 65 obtained from the Sepharose 6B column were pooled and heated at 90°C for 20 min. The heat-stable supernatant was fractionated by chromatography on hydroxylapatite. The hydroxylapatite column (2.5 x 20)

was equilibrated with 5 mM sodium phosphate (pH 6.8), washed with the same medium and the active fraction was eluted with 50 mM sodium phosphate. The active fraction was further purified by gel filtration on Sephadex G-75 which had been previously equilibrated with 10 mM Tris-HCl (pH 7.1).

Assay for histone deacetylase activity.

The assay for enzymatic deacetylation of histone follows the method proposed by Inoue and Fujimoto (15). Aliquots of the fractions to be tested (400 µl) were added to 25 µl portions of isotopically labeled histone solutions (58,800 cpm) and incubated at 37°C for 60 min. Reactions were terminated by the addition of 25 µl of concentrated HCl. The released ^{3}H-acetic acid was then extracted from the mixtures with 2 ml of ethyl acetate, and 1 ml of the organic solvent phase was counted in Bray's scintillation fluid (16). Radioactivity was measured in an Intertechnique model SL 30 scintillation spectrometer. The radioactive substrates used for histone deacetylase assays were prepared by incubating testicular cells in the presence of ^{3}H-acetate. Fresh rooster testis (50 g) were minced with scissors and gently dissociated with a syringe in 5 volumes of minimum essential medium (Eagle). The cell suspension was filtered, diluted to a final volume of 250 ml and cycloheximide was added to a final concentration of 0.2 mM. The cell suspension was incubated with 5 mCi of ^{3}H-sodium acetate (Amersham, 300 mCi/mmol) at 37°C for 1 hour. Following incubation histones were extracted from chromatin as previously described (12) except that all media contained 6 mM sodium butyrate.

Histone deacetylase activator assay.

The assay mixture contained 100 µg of activator deficient histone deacetylase and different amounts of the activator solution. After incubation for 60 min at 37°C, histone

deacetylase was assayed by the procedures previously defined.

Isolation of HMG protein 17 and assay with histone deacetylase.

The high mobility group proteins were prepared from rooster testis cell nuclei by the method of Goodwin et al.(17). The chromatin was extracted three times with 0.35 M NaCl, 10 mM Tris (pH 7.4), 1 mM PMSF. Proteins were then fractionated on the basis of their solubility in trichloroacetic acid. The proteins soluble in 0.35 M NaCl were treated successively with 2 per cent trichloroacetic acid (TCA) to precipitate the low mobility group and with 10 per cent TCA to remove HMG-1 and HMG-2. The remaining HMG-14 and HMG-17 were precipitated in 25 per cent TCA. The precipitate was redisolved in 7.5 mM sodium borate (pH 8.8), 0.15 M NaCl and purified HMG-17 was obtained by chromatography on CM-Sephadex C-25 (18). The assay of HMG-17 with histone deacetylase was performed as indicated in the previous paragraph. In some experiments HMG's 14 and 17 were obtained and assayed with histone deacetylase in rooster testis cell nuclei as described by Reeves and Candido (2).

Enzymatic hydrolysis.

Tryptic digestion was performed for 30 min. at 37°C with 100 μg/ml trypsin (Merck) and soybean trypsin inhibitor (Merck) was then added to 500 μg/ml. Pronase digestion (Merck, 25 μg/ml) was carried out at 37°C for 60 min. at pH 7.3. The incubation was terminated by boiling for 15 min. Control experiments showed that this treatment was sufficient to destroy completely the protease activity without affecting the histone deacetylase activator. Treatment with ribonuclease (Merck, 85 μg/ml) was at 37°C for 18 hours at pH 7.3.

Analytical techniques.

Amino acid analyses were carried out using a Beckman amino acid analyzer after hydrolysis of the samples in 6 N

HCl at 110°C for 24 hours. No corrections were made for hydrolytic losses. Proteins were analyzed electrophoretically on 15 per cent acrylamide slab gels by the method of Panyim and Chalkley (19), and in exponential polyacrylamide-SDS gels (10 %-16 %) as described by O'Farrell (20). The gels were scanned with a model 2410 Gilford linear transport scanner. Protein was determined by the procedure of Lowry et al. (21).

RESULTS

Activator deficient histone deacetylase

When purified nuclei from rooster testis cells were incubated with DNase I under conditions known to preferentially destroy active genes, about 10 per cent of the chromatin DNA was released. The supernatant obtained after DNase I digestion showed histone deacetylase activity. In order to separate the enzymatic activity from putative regulators, the

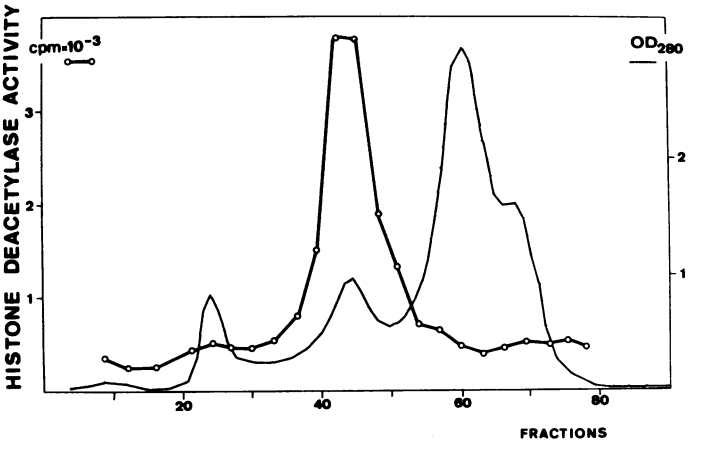

Figure 1: Sepharose 6B chromatography of the soluble material released after DNase I digestion of rooster testis cell nuclei. The sample was applied to a 2.5 x 100 cm column and eluted in 0.025 M Tris HCl buffer, pH 7.3, at a flow rate of 25 ml per hour. (——): A_{280}; (-o-o-): histone deacetylase activity.

nuclear extract was fractionated by gel filtration on Sepharose 6B. The separation achieved is ilustrated in Fig. 1, which shows the position of histone deacetylase activity. The fractions containing the enzymatic activity were pooled and stored at -40°C.

Histone deacetylase activator.

Preparation of the histone deacetylase activator from the nuclear DNase I digested material was facilitated by the fact that the activator, unlike the enzyme, is heat-stable. When the DNase I digested material was incubated for 20 min. at 90°C and then rapidly cooled to 0°C, most of the proteins floculated and after centrifugation the supernatant contained the histone deacetylase activator without enzymatic activity. When the fractions eluted from the Sepharose 6B column were treated in a similar way, the activator was detected in fractions 57 to 65 (Fig. 1). Assaying the activator with activator deficient histone deacetylase, the data plotted in Fig. 2 were obtained. The constant maximal level attained

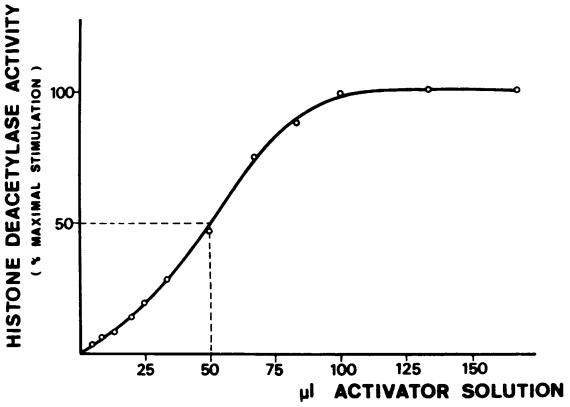

Figure 2: Determination of relative activity of heat-stable extract on activator-deficient histone deacetylase. Fractions 57 to 65 obtained from Sepharose 6B chromatography were pooled, heated and the supernatant assayed for activity as described in the text.

corresponded to a 4 fold stimulation of the basal activity. One unit of activator is defined as the amount (µg) of heat-treated extract required to stimulate activator deficient histone deacetylase to a 50 per cent maximal stimulation.

The heat-stable supernatant was fractionated by chromatography on hydroxylapatite. The active fraction eluted with 50 mM sodium phosphate was further purified by gel filtration on Sephadex G-75. The electrophoresis in SDS-acrylamide gels of the different steps of purification is shown in Fig. 3. The specific activity of the purified histone deacetylase activator is 510 units/mg of protein.

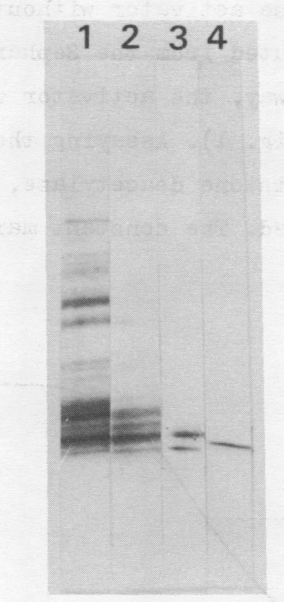

Figure 3: Comparative polyacrylamide gel electrophoretic patterns of different fractions obtained during the purification of the histone deacetylase activator. Slot 1 displays the proteins from the active fraction obtained after Sepharose 6B chromatography. Slot 2 displays the proteins of the heat stable supernatant. Slot 3 displays the active fraction obtained from the hydroxylapatite column. Slot 4 displays the histone deacetylase activator after chromatography on Sephadex G-75.

The activator is a non-dialysable molecule (Spectrapor 3 tubing, Spectrum Medical Industries), trypsin and pronase resistant and ribonuclease resistant. The molecular weight of the activator is 9,000 by gel filtration and by SDS-electrophoresis (Fig. 4 and 5). We have performed amino acid analysis of the purified histone deacetylase activator and the results are summarized in Table I. In this table the amino acid composition of the histone deacetylase activator and of the protein ubiquitin (22) are compared.

The molecular weight, chromatographic bevahior, thermal

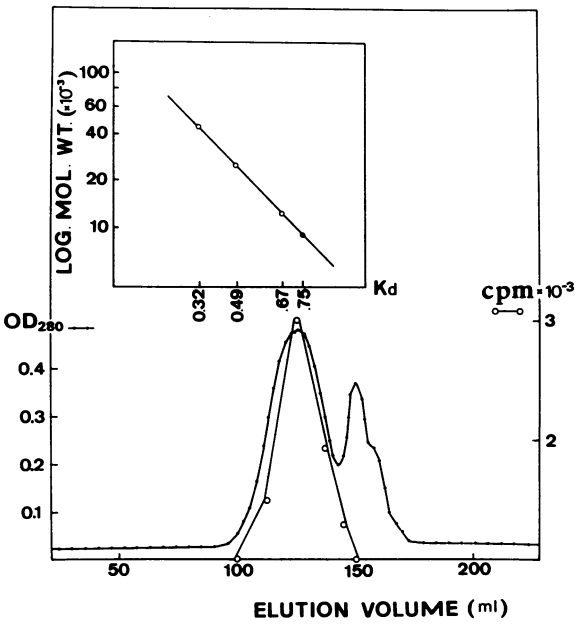

Figure 4: Gel filtration analysis of the histone deacetylase activator. The active fraction eluted from the hydroxylapatite column was introduced into a column of Sephadex G-75(1.5x100cm). The column was equilibrated and eluted with 10 mM Tris-HCl (pH 7.1). The eluate was monitored for histone deacetylase activator (o—o—o). Insert : Molecular weight determination. Marker proteins (open circles) : ovalbumin, chymotrypsinogen and cytochrome c. The closed circle indicates the elution position of the histone deacetylase activator.

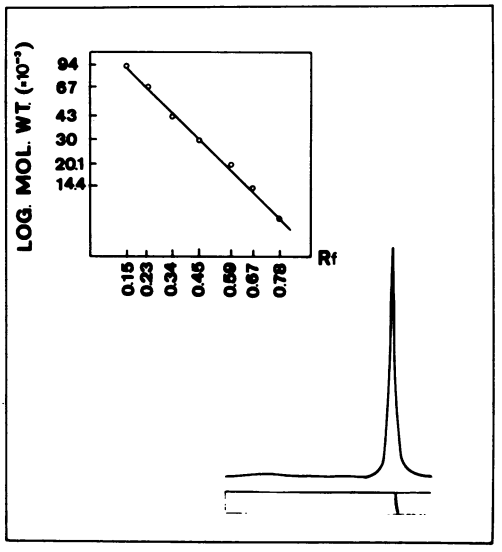

Figure 5: SDS-polyacrylamide gel electrophoretic analysis of the histone deacetylase activator. The active fraction eluted from the Sephadex G-75 column was analyzed by SDS-polyacrylamide gel electrophoresis. The electrophoretic mobility of the single band of the gel is 0.78. Insert : Molecular weight determination. Marker proteins (open circles): phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and α-lactalbumin. Closed circle: histone deacetylase activator.

stability, resistance to proteases and amino acid analysis of the histone deacetylase activator are very similar to the properties of the protein ubiquitin. To further probe the possible identity of this protein we have purified ubiquitin by two different procedures and assayed the protein with activator deficient histone deacetylase. Rooster testis ubiquitin was purified from the high mobility group nonhistone proteins by chromatography on Bio-Rex 70 (23) or by gel filtration on Sephadex G-75 (24). In both cases ubiquitin stimulated the enzymatic activity in vitro to a maximal level 4-times higher of that obtained with activator deficient histone deacetylase.

TABLE I

Amino acid composition of purified histone deacetylase activator and calf thymus ubiquitin

Amino acid	Ubiquitin	Histone deacetylase activator
Asp	6.97	6.85 (7)
Thr	7.10	6.03 (7)
Ser	3.40	4.21 (3)
Glu	12.56	11.92 (12)
Pro	3.26	3.32 (3)
Gly	3.85	4.53 (4)
Ala	2.00	2.85 (2)
Cys	0.00	0.00 (0)
Val	3.91	4.23 (4)
Met	0.73	1.48 (1)
Ile	6.49	5.36 (7)
Leu	9.20	7.85 (9)
Tyr	0.99	1.31 (1)
Phe	1.84	2.02 (2)
His	0.94	1.20 (1)
Lys	7.19	6.74 (7)
Arg	3.89	3.81 (4)
Trp	0.00	0.00 (0)

The data are presented as numbers of residues per molecule. The molecular weight of the histone deacetylase activator is assumed as 9,000. No corrections were made for hydrolytic losses. Numbers given in parentheses are the number of residues obtained from the sequence result. The amino acid composition of calf thymus ubiquitin is from (22).

Effect of HMG-17 on histone deacetylase activity.

The high mobility group nonhistone proteins were isolated from rooster testis chromatin by the method of Goodwin et al. (17). Proteins were fractionated on the basis of their solubility in trichloroacetic acid. Pure HMG-17 was obtained from the fraction soluble in 10 per cent TCA by chromatography on CM-Sephadex C-25 (Fig. 6). The electrophoresis of the peak eluted at 0.3 M NaCl and of the 10 per cent TCA soluble fraction are shown in Fig. 6 and Fig. 7 , respectively. We

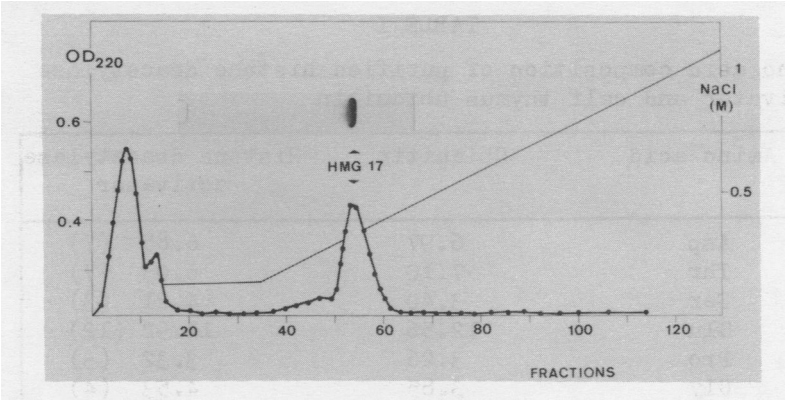

Figure 6: CM-Sephadex C25 chromatography of 10 per cent TCA soluble HMG proteins. Partially purified HMG proteins were applied to a 2.5 x 16 cm column of CM-Sephadex C25 equilibrated with 7.5 mM sodium borate buffer (pH 8.8). The polyacrylamide gel electrophoresis of the peak eluted at 0.3 M NaCl shows a single band with the mobility of HMG-17. (—•—) : A_{220} ; (——) : NaCl concentration.

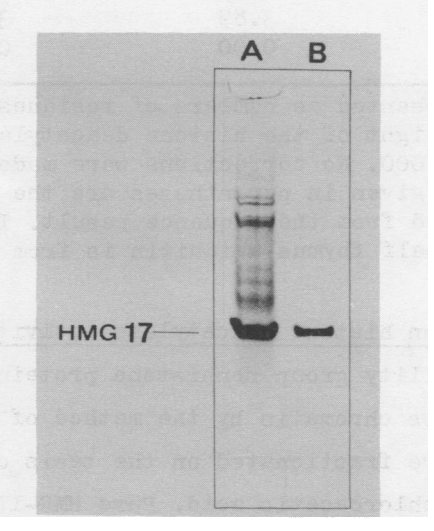

Figure 7: Comparative polyacrylamide gel electrophoretic patterns of different amounts of partially purified HMG-17. Total HMG proteins were fractionated on the basis of their solubility in trichloroacetic acid. The fraction soluble in 10 % TCA shows HMG-17 as the main component (B) . When the gel is overloaded, several contaminants appear (A).

have performed amino acid analysis of purified HMG-17 and the results are shown in Table II. In this Table the amino acid composition of the HMG-17 from rooster testis is compared with the known amino acid composition of chicken erythrocyte HMG-17 (25).

Reeves and Candido (2) have reported partial inhibition of histone deacetylase by HMG-14 and HMG-17. We have assayed purified HMG-17 with activator deficient histone deacetylase and the 10 per cent TCA soluble HMG proteins (mainly HMG-17) with purified nuclei. In both cases we could not detect the reported inhibitory effect of HMG-14 and HMG-17 on histone deacetylase (Table III).

TABLE II

Amino acid composition of isolated HMG proteins from rooster testis cell nuclei and nuclei of chicken erythrocytes (mol%)

Amino acid	HMG-17 testis	HMG-17 erythrocyte
Asp	11.2	9.1
Thr	2.0	3.0
Ser	3.2	4.3
Glu	12.2	11.7
Pro	11.6	12.1
Gly	9.9	10.0
Ala	16.7	17.2
Cys	-	-
Val	2.2	2.2
Met	-	-
Ile	-	-
Leu	1.2	1.2
Tyr	-	-
Phe	-	-
His	0.2	0.2
Lys	25.0	23.6
Arg	4.6	4.6

No corrections were made for hydrolytic losses.
The amino acid composition of chicken erythrocyte HMG-17 is from (25).

TABLE III

Effect of 10 per cent TCA soluble HMG proteins and purified HMG-17 on histone deacetylase activity assayed *in vitro*

Assay with activator deficient histone deacetylase (a)					
	Protein concentration (µg/ml) per reaction				
	None	40	80	160	360
10% TCA soluble HMG proteins	4,625	4,610	4,717	4,653	5,041
Purified HMG-17	4,552	4,324	4,643	5,007	-
Assay with nuclei isolated from testis cells (b)					
	Protein concentration (µg/ml) per reaction				
	None	90	180	360	730
10% TCA soluble HMG proteins	7,480	7,330	7,740	7,928	7,854

Histone deacetylase activity is listed in the table as the amount of ^{3}H-acetate (cpm)released from the acetylated histones.
(a) The conditions of the assay are described in Materials and Methods.
(b) The conditions of the assay are described in reference (2).

DISCUSSION

We report here the isolation from rooster testis cell nuclei of a heat-stable activator of histone deacetylase. Many of its properties coincide with those of the protein ubiquitin. Moreover, ubiquitin isolated from testis cell nuclei by two different procedures stimulates histone deacetylase *in vitro*.

Ubiquitin is a small highly stable globular acidic protein, widely distributed in prokaryotes and eukaryotes , with an amino acid sequence highly conserved during evolution (26-28). One of the known functions of ubiquitin is the ATP dependent breakdown of proteins to amino acids (29). The substrates of this proteolitic system are covalent conjugates

of proteins and ubiquitin.

Ubiquitin is present in chromatin in two different forms : covalently conjugated and in the free state. Free ubiquitin is localized in a domain of chromatin enriched in DNA sequences which can be transcribed into cellular RNA (23,30) and there is some evidence that increases in free ubiquitin are associated with increased gene activity (31).

We may speculate that if ubiquitin has *in vivo* the same stimulatory activity on histone deacetylase than *in vitro* , this protein might be responsible for the high turnover (half life of 3 to 7 min) of acetyl groups on the core histone of a small sub-set of nucleosomes comprising no more than 10 to 15 per cent of chromatin (11). The bulk of acetylated histones (40 per cent of H3 and H4) is deacetylated at an approximately 10-fold slower rate (11).

Reeves and Candido (2) considered the possibility that within the regions of transcriptionally active chromatin an endogenous inhibitor of the histone deacetylase is selectively localized. They suggested that the proteins of the nonhistone High Mobility Group, HMG-14 and HMG-17, present in active chromatin, partially inhibit the deacetylase enzyme.

We investigated if HMG-17 from rooster testis cells could inhibit histone deacetylase in our system. We have shown that HMG-17 does not inhibit histone deacetylase assayed *in vitro*. In addition, the HMG's 14 and 17 from rooster testis isolated and assayed as described by Reeves and Candido (2) show no effect on nuclear deacetylase activity.

Our results agree, however, with those described by Reeves and Candido (2) for the trout specific protein H6. This protein has many similarities to calf thymus HMG-17 (32). Protein H6, present in active genes, does not cause

any inhibitory effect on histone deacetylase.

Further studies are necessary to probe if ubiquitin has stimulatory activity on histone deacetylase *in vivo* and to determine the relationship between gene activity , rapid turnover of acetyl groups in the core histone of a small sub-set of nucleosomes and the localization of ubiquitin in certain domains of chromatin.

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