Studies of Acetylation and Deacetylation in High Mobility Group Proteins

IDENTIFICATION OF THE SITES OF ACETYLATION IN HIGH MOBILITY GROUP PROTEINS 14 AND 17

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Duck erythrocytes were incubated with [3H]acetate both in the presence and absence of sodium butyrate. Subsequent perchloric acid extraction of the nuclei, followed by selective acetone precipitation, CM-Sephadex ion exchange chromatography, and gel filtration yielded radioactively labeled high mobility group (HMG) proteins HMG-14 and HMG-17 in pure form.

Extensive enzymatic degradation of the proteins followed by amino acid analysis of the digests yielded a significant amount of material eluting in the position of \( \epsilon-N \)-acetyllysine. Furthermore, automated Edman degradation of intact \[^{14}C\]acetate-labeled HMG-14 and HMG-17 identified the specific sites of acetylation of these proteins. In both erythrocyte HMGs isolated from cells not exposed to butyrate, the lysine residue at position 2 was the only one found to be labeled. However, one additional site in HMG-14 and two additional sites in HMG-17 were found in the proteins from cells incubated in butyrate.

Finally, studies of the enzymatic deacetylation of HMG-14 and HMG-17 confirmed that both nuclear proteins serve as deacetylase substrates and that butyrate inhibits their deacetylation, just as in the case of other HMG proteins and nucleosomal core histones.

In recent years, the search for nuclear proteins which have specific regulatory functions in chromatin structure and gene expression has focused on a group of nonhistone proteins of characteristic solubility and electrophoretic properties called the high mobility group or HMG\(^*\) proteins. Data showing very strong interspecies conservation of primary structures of the HMG proteins (1–3), as well as a rather large number of copies of the proteins per nucleus (4), strongly suggest that the HMG proteins do not have a specific role in controlling the activation of transcription from unique DNA sequences. However, a number of recent findings indicate that HMG proteins, and particularly HMG-14 and HMG-17, are intimately associated with active chromatin, and suggest that the presence or absence of these proteins helps determine whether the DNA within the nucleosome exists in a template-active or inactive form.

Limited DNase I digestion of avian erythrocyte chromatin under conditions which have been shown to degrade selectively the active or potentially active genes (5–7) causes the selective release of HMG proteins (8). This phenomenon has been observed as well in trout testes (7) and rabbit thymus (9) chromatin. Other data have indicated that, relative to inactive chromatin, actively transcribing chromatin is enriched in HMG proteins (10). More recently, HMG-14 and HMG-17 have been specifically implicated in conferring DNase I sensitivity to chromatin and are associated with nucleosomes containing transcribed DNA sequences (11–14). Furthermore, recent data have indicated that HMG-14 and HMG-17 can partially inhibit the activity of protein deacetylases toward histone substrates (15). These findings are of particular interest because much evidence has been compiled implying a correlation between gene activity and the degree of acetylation of the nucleosomal core histones (16–21), as well as a direct correlation between levels of histone H3 and H4 acetylation and the accessibility of the associated DNA sequences to DNase I (22–25).

Given the apparent close association of HMG-14 and HMG-17 with transcriptionally active nucleosomes and the potential connections which have arisen between these proteins and histone acetylation, it was our intention to determine whether HMG-14 and HMG-17 are themselves subject to posttranslational acetylation and deacetylation, as are the histones (16, 26) and other proteins of the HMG class (27, 28), and, if so, to locate the actual sites of modification. In addition, we have examined whether HMG-14 and HMG-17 are substrates for deacetylase activity, and whether sodium butyrate inhibits their deacetylation as it does for histones.

**Experimental Procedures**

Radioactive Labeling and Isolation of HMG Proteins—Fresh Pekin duck blood was centrifuged for 10 min at 7000 \( \times \) g and the plasma decanted. To 500 ml of packed erythrocytes was added 200 ml of pH 7 buffer containing 10 mm Tris-HCl, 10 mm NaCl, 3 mm MgCl\(_2\) and 1 mm CaCl\(_2\). Finally, 50 mCi of high specific activity \[^{3}H\]acetate (10 Ci/mmol) was added and the mixture was incubated for 1 h at 37 \( ^\circ \)C with shaking. A second 500-ml portion of packed erythrocytes was suspended in the same buffer described above except that it was also 50 mm in Na-butyrate. These cells were incubated with 50 mCi of lower specific activity \[^{3}H\]acetate (100 mCi/mmol) for 6 h at 37 \( ^\circ \)C with stirring and aeration. Both cell suspensions were subsequently treated in the same manner. The erythrocyte nuclei were isolated as described previously (29). The nuclei were extracted with 5% perchloric acid and the HMG proteins were separated from other proteins by selective acetone precipitation (30). The total HMG proteins thus obtained were fractionated by ion exchange chromatography on CM-Sephadex (29). The HMG-14 and HMG-17 fractions were freed from small amounts of contaminants by passage through a column (2 \( \times \) 60 cm) of Sephadex G-50 equilibrated in 0.02 m HCl. Fractions of 5.5 ml were collected at a flow rate of 20 ml/h. The purity of the two proteins was assessed by SDS-polyacrylamide gel electrophoresis, amino acid

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Identification of e-N-Acetyllysine and Sequence Analysis—The purified proteins were hydrolyzed by successive treatments with trypsin and pronase, and e-N-acetyllysine was separated from other amino acids in the digest as described previously for the analysis of other HMG proteins (27). Primary structural data were obtained by automated Edman degradation as described earlier (28).

Deacetylation of [\(^{3}H\)]acetate-labeled HMG Proteins—Protein deacetylation was prepared from calf thymus nuclei according to procedures described previously (26). An efficient microassay method (31) was used to monitor the release of [\(^{3}H\)]acetate from HMG and histone substrates in the presence of deacetylase. The effect of butyrate on the deacetylation of the same HMG substrates was assayed in the same way, except that the incubation medium in all cases contained 10 mM sodium butyrate.

Materials—Fresh calf thymus glands were purchased from Max Cohen, Newark, NJ. Fresh duck blood was a generous gift from Long Island Duck, Inc., Eastport, NY. Sodium [\(^{3}H\)]acetate was purchased from New England Nuclear. Trypsin was obtained from Worthington and Pronase from Calbiochem. Reagents necessary to carry out Edman degradations and high pressure liquid chromatography were obtained from Beckman Instruments and from Burdick and Jackson. All other reagents were of the highest purity available.

RESULTS

Radioactive Labeling and Isolation of HMG Proteins—The HMG proteins were prepared from duck erythrocytes which had been incubated with sodium [\(^{3}H\)]acetate in the presence and absence of sodium butyrate. A final purification step employing Sephadex G-50 gel filtration proved to be an effective method for preparing either HMG-14 or HMG-17 from contaminating proteins which remained in small amounts following CM-Sephadex chromatography. The result of SDS-polyacrylamide gel electrophoresis of the purified proteins is shown in Fig. 1.

The pure preparations of HMG-14 and HMG-17 from unbutyrated erythrocytes were found to have incorporated significant amounts of [\(^{3}H\)]acetate; HMG-14 was labeled to the extent of 12,000 dpm/mg (approximately 125 dpm/nmol), while HMG-17 had a somewhat higher activity: 18,000 dpm/mg (approximately 165 dpm/nmol). In comparison, HMG-1 isolated from the same erythrocyte nuclei contained 30,000 dpm/mg (750 dpm/nmol) and HMG-E contained 16,000 dpm/mg (400 dpm/nmol). In further comparisons of [\(^{3}H\)]acetate incorporations into different erythrocyte nuclear proteins, HMG-1, the HMG protein found to have the highest specific activity, was approximately 20% as active as the total histone fraction (both expressed in dpm/mg).

HMG proteins isolated from erythrocytes incubated in butyrate showed far greater incorporations of [\(^{3}H\)]acetate than did proteins from unbutyrated cells. HMG-1 had a specific radioactivity of 3000 dpm/nmol and HMG-E contained 2000 dpm/nmol. HMG-14 and HMG-17 showed even greater percentage increases, their specific activities being 1500 and 2200 dpm/nmol, respectively. These both represent increases well in excess of an order of magnitude.

Identification of e-N-Acetyllysine in HMG-14 and HMG-17—The purified, [\(^{3}H\)]acetate-labeled HMG-14 and HMG-17 preparations were subjected to extensive trypsin and pronase digestion, as described in previous studies of the acetylation of calf thymus HMG-1 and HMG-2 (27). Subsequent amino acid analysis and monitoring of radioactivity revealed that the HMG-14 and HMG-17 digests both contained a ninhydrin-positive radioactive peak corresponding exactly in elution position to that of e-N-acetyllysine.

Identification of the Sites of Acetylation in HMG-14 and HMG-17—Automated Edman degradation through the first 17 amino acids of [\(^{3}H\)]acetate-labeled erythrocyte HMG-14 and HMG-17 revealed distinct differences between the proteins from butyrated and unbutyrated cells. Only a single site, Lys-2, was radioactively labeled in HMG-14 and HMG-17 isolated from unbutyrated cells. Based on an initial yield of 50% (relative to the amount of protein originally taken for sequencing) and a repetitive yield of 94%, specific activities of 150 and 200 dpm/nmol were calculated for the sites in the respective proteins. In proteins from butyrated cells, on the other hand, two sites, Lys-2 and Lys-4, were labeled in HMG-14, and three sites, Lys-2, Lys-4, and Lys-10, contained [\(^{3}H\)]acetyl groups in HMG-17. For HMG-14, the calculated respective specific activities for Lys-2 and Lys-4 were 900 and 200 dpm/nmol, and for HMG-17, the lysines at positions 2, 4, and 10 had incorporations of 700, 100, and 1200 dpm/nmol, respectively. Following Edman degradation, the residual poly-peptide chains were found to have virtually none of the radioactive acetyl remaining.

A summary showing the sites of acetylation of several HMG proteins and nucleosomal core histones is presented in Fig. 2. Comparison of the sequence data presented in Fig. 2 with data from an earlier study (3) also shows that, with one exception (position 17 in HMG-14), the NH\(_2\)-terminal primary structures of the corresponding proteins in duck and chicken erythrocytes are identical. These structures, in turn, are very homologous to (but not identical with) those reported earlier for the calf thymus proteins (32, 33).

Enzymatic Deacetylation of Radioactive HMG Proteins—The ability of [\(^{3}H\)]acetate-labeled HMG proteins to serve as substrates for calf thymus deacetylase was tested by incubating the various HMG preparations with the purified enzyme for periods ranging from 0 to 60 min and measuring the release...
of [3H]acetate. The rates of deacetylation of erythrocyte HMG-14 and HMG-17 were compared to those of HMG-1, HMG-E, and histone H4 under the same conditions. The data obtained for HMG-1 and histone H4 were virtually identical to those reported earlier (28), with 40% and 92% of the total counts released, respectively, after a 1-h incubation with the deacetylase. Fifty per cent of the total [3H]acetate counts were released from HMG-E, a figure comparable to that seen earlier for the deacetylation of HMG-2 (28). HMG-14 and HMG-17 also served as substrates and were deacetylated to the extent of 60% after 1 h. The significantly higher rate of HMG-14 and HMG-17 deacetylation, as compared to the rates found for HMG-1 and HMG-E, was observed consistently. Furthermore, the presence of 10 mM sodium butyrate inhibited the deacetylation of all the duck erythrocyte HMG proteins as well as histone H4. Again, the per cent inhibition for HMG-1 and H4 checked well with previous observations (28), being 55% and 75% respectively, and for HMG-E the corresponding figure was 65%. Butyrate inhibition of deacetylase activity, using HMG-14 and HMG-17 as substrates, was approximately 70%. Table I summarizes the results for the deacetylation of various substrates in the presence and absence of 10 mM sodium butyrate.

**DISCUSSION**

The data presented in several sets of experiments establish that HMG-14 and HMG-17 are subject to posttranslational acetylation of lysine ε-amino groups, similar to that observed in the other HMG proteins and in the histones of the nucleosome core. The incorporation of [3H]acetate into erythrocyte HMG-14 and HMG-17 and the subsequent isolation of ε-N-[3H-acetamido]lysine in these proteins confirm the nature of the modification, and the conclusion is further supported by the fact that enzymatic deacetylation releases the modifying group. Finally, the actual sites of modification were identified by automated Edman degradation of the [3H]acetate-labeled proteins.

Primary sequence analysis of duck erythrocyte HMG-14 and HMG-17 after labeling with [3H]acetate in the absence of butyrate revealed that both proteins are acetylated at a single lysine residue in the NH₂-terminal of the molecule, namely, Lys-2. However, similar analyses of the same proteins labeled in the presence of butyrate revealed labeling at multiple sites in both proteins. In the case of HMG-14, two of the four possible sites in the NH₂-terminal portion were labeled, namely Lys-2 and Lys-4. Three of the six possible sites of modification in the NH₂-terminal region of HMG-17 (Lys-2, Lys-4, and Lys-10) were found to have [3H]acetate activity under these conditions.

The distinct differences between quantity and pattern of acetylation in HMG proteins from cells incubated in the presence or absence of sodium butyrate lead to several noteworthy observations. In the first place, the inhibition of deacetylase activity due to the presence of butyrate results in a tremendous increase not only in the number of sites but also in the number of HMG molecules incorporating and retaining [3H]acetate. Taking into account both the increase in specific activity of the substrate HMG proteins and the much lower specific activity of the [3H]acetate used as a precursor in the butyrate-treated cells, one sees that butyrate treatment results in an increase of 3 orders of magnitude in the percentage of sites radioactively labeled in HMG-14 and HMG-17. Although some HMG molecules may well be multiply acetylated, the data suggest that as many as 1 in 100 HMG molecules incorporated [3H]acetate under these conditions (and one does not take into account molecules that are acetylated with nonradioactive acetate). These findings are in agreement with an earlier study (34) in which it was observed that deacetylase activity is significantly greater in mature avian erythrocytes than in earlier, immature stages of erythroid development. Such high levels of deacetylase activity would be expected to complicate studies of [3H]acetyllysine formation in the HMG proteins as well as in erythrocyte histones. While butyrate inhibition of deacetylase activity results in greatly enhanced 3H activities in all of the HMG proteins, the greatest effects seem to be with regard to HMG-14 and HMG-17. Whether or not this apparent heightened sensitivity of HMG-14 and HMG-17 to deacetylase activity is connected to their localization in more accessible regions of chromatin and to their putative role in modifying the structure of the nucleosome core remains to be determined.

Of further interest are the multiple sites of acetylation and their differing susceptibilities to acetyl group turnover. Lys-2 is significantly labeled and is the only site containing detectable amounts of 3H activity after labeling in the absence of Na-butyrate. As noted, its activity is greatly increased in butyrate-treated cells. This suggests that Lys-2 is a site of rapid acetyl group turnover, a site for which acetyl transferase has a high enough affinity to effect [3H]acetate incorporation at a rate which exceeds the rate of acetyl group removal by the active deacetylases of the mature red cell. Lys-4 in both HMG-14 and HMG-17 is minimally labeled and then only in the presence of butyrate, suggesting that this site is one of very low acetyl group turnover. Lys-10 in HMG-14 provides a striking contrast to the other sites. It is not labeled at all in the absence of butyrate, but has a specific activity exceeding that of all other sites, even Lys-2, when butyrate is present. It seems possible then that Lys-10 is a site of very rapid acetyl group turnover, but also one which is particularly susceptible to deacetylase activity.

**Studies of the in vitro deacetylation of HMG-1, HMG-E, and histone H4, used as standard substrates, gave data virtually identical to that reported in earlier studies (28). With these points of reference in hand, HMG-14 and HMG-17 were consistently observed to be highly susceptible to deacetylase activity. All of the HMG proteins, then, were found to be deacetylated, but at a rate lower than that seen for histone...
H4. Furthermore, consistent differences were observed between the various HMG proteins both in their susceptibility to deacetylase action and in butyrate inhibitory effects on deacetylase activity. These observations of a dynamic turnover of acetyl groups at multiple sites in the DNA-binding domains of the HMG proteins are in keeping with a model in which postsynthetic acetyl group transfer reactions play a role in determining DNA and nucleosome conformations.

The HMG proteins in this study were isolated from intact avian erythrocytes because these cells were found to afford higher incorporations of [3H]acetate than did a calf thymus histones. Incorporations of [3H]acetate in the basic NH2-terminal regions (28, 35-37) correlates well with a relaxation of constraints upon the most likely DNA-binding domains. The acetylation of the core histones subject to acetyl group transfer and have their sites of acet-...

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