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HIGH MOBILITY GROUP PROTEINS FROM CHO CELLS AND THEIR MODIFICATIONS DURING CELL CYCLE.

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SUMMARY A micromethod was established to extract the HMG proteins from whole CHO cells and their enzymic modifications at interphase and metaphase were determined. Phosphorylation of all four HMG proteins was observed during interphase with HMG-17 as the predominant phosphate acceptor. Metaphase cells exhibited quite significant changes of the phosphorylation pattern: the specific phosphorylation of HMG-17 decreased to half the interphase value, whereas that of the other HMG proteins increased by a factor of three.
Acetylation of the HMG proteins could not be detected in CHO cells, neither during interphase nor metaphase.

INTRODUCTION The high mobility group proteins (HMG 1,2,14 and 17) are a subclass of chromosomal proteins of general occurence in the eukaryotic kingdom (1-3). Their association with nucleosomes and the presence of HMG-14 and -17 in actively transcribed regions of the chromatin is indicative of their role in chromation organization or control of gene expression. These findings led to the expectation that the HMG proteins in vivo undergo similar post-translational modifications as it is known for histones; it is further likely that the degree of the expected modification changes during the cell cycle as in the case of the histones H1 and H3 (4).

Very recently it was shown by in vitro experiments with different tissues that all the HMG proteins were acetylated (5), HMG-1,2 methylated (6) and HMG-14 and -17 phosphorylated (7,8). To elucidate whether the four HMG proteins are substrates for kinases and acetyltransferases in the living cell, a CHO culture was grown in the presence of the respective radioactive precursors and the modified proteins were analysed. In addition the change of the modifications during cell cycle was studied using interphase and metaphase cells.

MATERIAL AND METHODS

Cell growth and labeling conditions. Chinese hamster cells (line CHO from Seromed München) were grown as monolayers in Ham's F-10 medium supplemented with 10% calf serum, 5% fetal calf serum, penicillin and streptomycin. For the labeling experiments during interphase each monolayer culture in 10 ml medium was exposed to 1 mCi carrier-free P-32 phosphoric acid (50-140 Ci/mg P), or 1 mCi C-14 acetic acid (54.2 mCi/mmol) plus 7 mM butyrate, respectively for 2h prior to the isolation of the high mobility group proteins. After decanting the medium,

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interphase cells were harvested by addition of hypotonic puffer and with the aid of a rubber policeman. The use of trypsin, which leads to an altered protein pattern was avoided. Metaphase arrested cells were obtained by treatment of the monolayer cultures with colcemid (0.06 µg/ml) for 1 h. Then the loosely attached cells were dislodged by gentle shaking and discarded. The monolayer was overlaid with 10 ml of fresh medium, containing colcemid (0.06 µg/ml) and the respective radioactive precursor, and incubated for additional 4h. For acetylation experiments the fresh medium further contained 7 mM sodium butyrate. Metaphase cells were harvested by shaking the culture bottle and collecting the cells by centrifugation. The mitotic index obtained was 90%.

Isolation and identification of the HMG proteins. All operations were carried out at 4° and all buffers contained 0.2 mM PMSF to prevent proteolytic degradation. In the case of the phosphorylated proteins the buffers were supplemented with 1 mM $ZnCl_2$ an inhibitor of phosphatase (9), isolation of the acetylated proteins was performed in the presence of 7 mM sodium butyrate to inhibited deacetylation (10). The collected cells were washed once with buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, 2 mM KCl_2, 1 mM $CaCl_2$ pH 7.4) (11). After centrifugation the cells were extracted four times with 0.5 ml 0.35 M sodium chloride followed by a 2% trichloroacetic acid (TCA) precipitation of the low mobility group proteins. These proteins were separated by centrifugation for 10 min at 40 000 g. The supernatant was made 20% of TCA (w/v) and the HMG proteins were spun down for 20 min at 40 000 g. Starting with about 10^6 cells, 12 µg HMG proteins were recovered.

The purity of the isolated HMG proteins was confirmed by gel electrophoresis and they were compared with the well characterized HMG proteins from chicken erythrocyte nuclei (11). First they were run on a SDS-acrylamide slab gel (12), after staining the individual protein bands were cut out and rerun on an acetic acid-acrylamide-urea gel (13). In order to determine the degree of phosphorylation, the protein species were separated by SDS gel electrophoresis. After staining with Coomassie Brillant Blue G-250 the bands were cut out, counted for radioactivity and the phosphate content for each protein fraction was calculated as described (14).

The determination of a possible acetylation of these proteins was performed as in the case of the phosphorylation. After staining the gels were treated with Enhance (New England Nuclear), dried in a slab gel dryer (Biorad Laboratories) and exposed at -70° to a Kodak X-ray film for 7-10 days. Acetate incorporation was estimated by scanning the film with a Gilford photospectrometer at 520 nm. The composition of the HMG protein mixture was determined by scanning a stained SDS gel and weighing the respective areas under the peaks.

RESULTS The modification of the conventional method (1) enabled us to isolate small quanities of HMG proteins from monolayer cultures of CHO cells without prior separation of cell nuclei. Fig.1A shows a SDS gel of the 0.35 M NaCl extracted and 20% TCA precipitated proteins in comparison with the well charactized HMG proteins from chicken erythrocyte nuclei (11). Fig. 1B reveals a scan of the HMG proteins in the acetic acid-urea gel after a preceding resolution in a SDS gel (cf. the legend of Fig. 1). It is obvious, that the electrophoretic mobilities of the HMG proteins from the CHO cells differ slightly from those of the chicken erythrocyte proteins, the magnitude of the differences depends on the gel system applied. The two larger proteins (HMG-1 and HMG-2) are less resolved than the respective chicken proteins in both gel systems, which may be indicative of a greater similarity of the CHO proteins. The four proteins isolated by our method from CHO cells met all criteria of HMG proteins: extractable with 0.35 M sodium chloride, soluble in 2% TCA, but precipitable with 20% TCA, and the electrophoretic mobilities (1). The relative abundancy of the individual HMG proteins within the mixture was determined to be 3.9:1:4.3

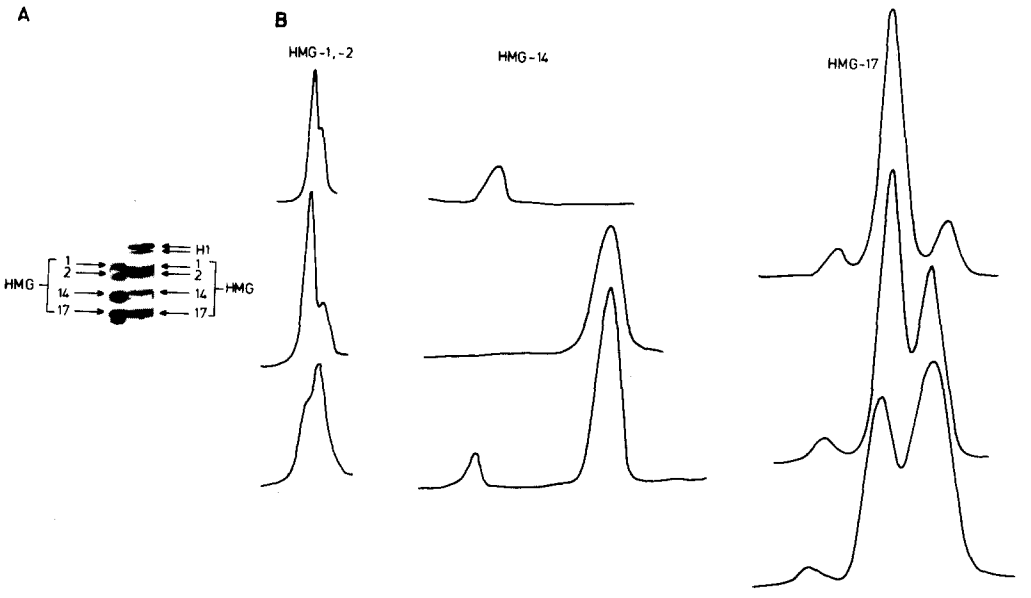

Fig. 1. A: SDS slab gel of CHO high mobility group proteins (right slot) compared with a synthetic mixture of equal amounts of the four erythrocyte HMG proteins (left slot).

B: The protein bands, dissected from the SDS gel were applied to acrylamide-urea-acetic gel electrophoresis is 15 cm long tubes. HMG-(1,2) from CHO cells (top) mixed with erythrocyte HMG-1 (middle) and mixed with erythrocyte HMG-2 (bottom). CHO HMG-14 (top), erythrocyte HMG-14 (middle) and a mixture of both (bottom). CHO HMG-17 (top) with increasing amounts of erythrocyte HMG-17 (middle and bottom). Direction of electrophoresis was from left to right.

for HMG-(1+2), HMG-14 and HMG-17 by scanning the stained protein bands and weighing the peak areas. As a control the isolation procedure of extracting whole cells was compared with preparation of cell nuclei and their subsequent extraction. The results obtained did not exhibited differences, neither in the amount of HMG proteins nor in the composition of their mixture. All preparations of the HMG proteins contained the histone H1, which is normally separated by acetone precipitation (15). In our case, the H1: HMG ratio was utilized as a marker for the reproducibility of the preparations.

The second part of this investigation is concerned with the question whether the HMG proteins are phosphorylated and acetylated in vivo as it is known for other chromosomal proteins (4,16,17) and if these modifications were modulated during the cell cycle. To investigate the phosphorylation, interphase CHO cells were grown in the presence of P-32 phosphoric acid for 2h. The HMG proteins were purified as described and applied to the SDS gel. After staining, the individual protein bands were cut out and counted for radioactivity. Because the HMG-1 and 2 were

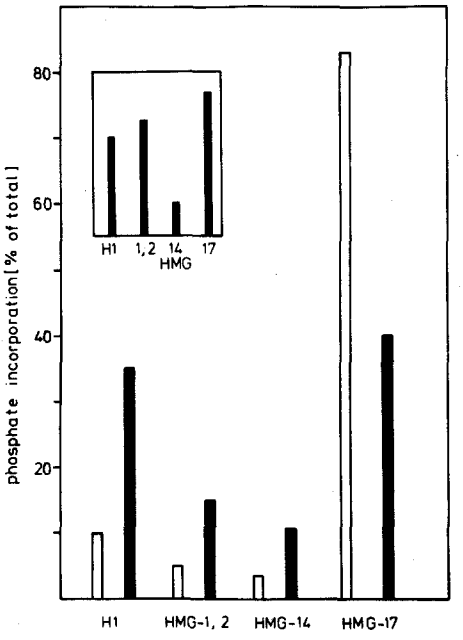

Fig. 2: Incorporation of phosphate into the four HMG-proteins and histone H1 from interphase cells (open bar) and metaphase cells (solid bar). Taking the sum of the counts (total incorporation) as 100%, the relative amount of phosphate was calculated for each protein component. The values represent an average of at least five protocolls. Insert: Relative abundancy of the four proteins and H1 found in interphase and metaphase CHO cells.

not fully resolved, they were cut out as one band and counted together. The results are shown in Fig. 2 (open bars). All four HMG proteins contain radioactive phosphate, however, the incorporation into HMG-17 is significantly higher than into the other species. Considering the relative mass ratio of the individual components in the mixture (insert in Fig. 2) the HMG-17 has also the highest specific radioactive phosphate content, followed by HMG-14, whereas HMG-(1+2) has the smallest value (Table 1).

TABLE 1
Phosphate content of the individual HMG proteins calculated per relative mass unit.

HMG component	Interphase	Metaphase
1+2	1.3	4.2
14	3.5	10.6
17	18.9	9.1

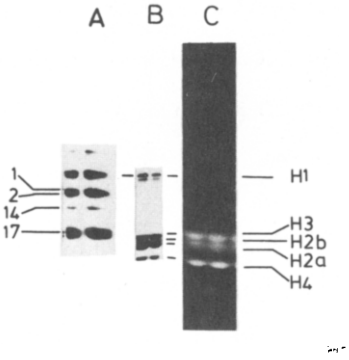

Fig. 3: SDS gel of interphase HMG proteins (A), histones (B) and autoradiogramm of histones (C) after exposure of the CHO cells to C-14 acetate in the presence of 7 mM sodium butyrate.

Metaphase arrested cells were prepared by colcemid treatment resulting in a mitotic index of 90%. The phosphorylation pattern of the metaphase HMG proteins are shown in Fig. 2 (solid bars). Again all four HMG proteins contain phosphate, but the pattern is quite different from that of the interphase cells. Differences, however, in the composition of the protein mixture could not be detected. Whereas the phosphate content of HMG-(1+2) and HMG-14 increased about threefold, it dropped to half the value of the interphase cells for HMG-17. Calculation of the specific phosphate incorporation (Table 1) makes it evident, that the two smaller proteins, HMG-14 and 17, are phosphorylated to the same extent during mitosis. The two larger proteins, HMG-1 and 2, reveal about half the value of HMG-14 or 17.

The acetylation of the HMG proteins has been reported to occur in vitro (5). To determine this modification in the CHO cells, monolayer cultures were grown in the presence of C-14 acetic acid and butyrate for 2 h prior to the isolation of interphase HMG proteins. They were purified and separated on a SDS gel and autoradiography was performed as described in the Experimental Section. However, no radioactive acetate could be detected in the HMG protein bands (Fig. 3). That an acetylation of chromosomal proteins takes place under the conditions employed, is documented by the positive autoradiography of the histone bands. The amount of radioactive precursor incorporated into the four core histones decreases in the following order: H4>H3>H2a, H2b. The linker histone H1 did not incorporate radioactive acetate, this is taken as an evidence that the acetic acid was only metabolized to modify the lysine side chains. The possibility, that the acetylation of the HMG proteins is only a mitotic event, was further studied. However, HMG

proteins extracted from metaphase arrested cells subsequent to incubation with C-14 acetate did not reveal any incorporation of radioactivity, whereas the core histones were again labeled. The distribution of radioactive acetate between the single histones was the same as that found with the histones of the interphase cells.

DISCUSSION. This is the first description and characterization of the high mobility group proteins from CHO cells. The proteins were compared with the known HMG proteins from chicken erythrocyte nuclei (11). The relative ratio of the individual components in the mixture is different from that found with chicken erythrocyte HMG proteins. In the CHO cells HMG-17 is the main component followed by HMG-(1+2) and HMG-14, whereas in chicken erythrocytes HMG-1 and 2 constitute the main part.

Our results further demonstrate, that the four HMG proteins 1,2,14 and 17 are phosphorylated in vivo in CHO cells during interphase and metaphase. The results agree with our own in vitro investigations (18) demonstrating that the four erythrocyte HMG proteins are substrates for two portein kinases, a cAMP-dependent and a cyclic nucleotide-independent nuclear enzyme. They disagree, however, with studies of other authors (7,8) who showed that only HMG-14 and HMG-17 are phosphorylated in vitro. Our own in vitro studies with the nuclear protein kinase (18) exhibited nearly the same phosphorylation pattern as found with the present in vivo phosphorylation in interphase cells; i.e. the HMG-17 is the predominant phosphate acceptor. The apparent specific incorporation of phosphate into the individual proteins as shown in Table 1, demonstrate differences between the interphase and metaphase cells. In the interphase cells HMG-17 is the predominant phosphate acceptor, whereas in the metaphase cells HMG-14 and HMG-17 are equally active. This may constitute a further result of cell cycle dependent regulations affecting the phosphorylation of chromosomal proteins, as it is known from the phosphorylation of the histones H1 and H3 (4).

Acetylation of the HMG proteins could not be detected in our experiments, neither in interphase nor in metaphase CHO cells. That modification has been reported to occur, however, in vitro with all four HMG proteins when a calf thymus homogenate is incubated with C-14 acetylCoA (19). It remains questionable therefore, whether those in vitro studies reflect a physiological event.

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