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AN OVERALL ANALYSIS OF PHOSPHORYLATED HISTONE VARIANTS IN CHO CELLS.

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SUMMARY

The histones are shown to be unequally extracted from nuclei or isolated chromatin of Chinese hamster ovary cells. To realise an overall study of phosphorylation of histone variants, we have developed an analytical system in which the five main histones are first separated on a sodium dodecyl sulfate gel then, after electroelution analysed on a Triton acid urea gel. Thus, no specific loss of histones is detected. In CHO cells, the following histone variants are phosphorylated : 5 % of the non acetylated and the monoacetylated H4 - around 30 % of H2A1 and H2A2 - less than 2 % of H2B1 - H31 and its monoacetylated form are the main phosphorylated variants of the three H3. All the H1 molecules are phosphorylated both in H11 and H12.

INTRODUCTION

It is now well established that individual histones can be modified by several post-translational alterations of aminoacid residues. Among them, the phosphorylation, acetylation, ubiquitine additions have been the most studied and their possible involvment in chromatin structure has been suggested (for review see 1, 2). On the other hand, histones 2A, 2B and 3 can be resolved into variants by polyacrylamide gel electrophoresis in presence of non ionic detergents (3, 4). The relative amount of the different variants was demonstrated to change during embryogenesis (5) and they may play a role in functional differentiation of various parts of chromatin (6).

For H1 and H2A many aspects of phosphorylation have been reported such as the determination sites of addition and the relative level

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of phosphorylation during the cell cycle (1, 7, 8). In this paper we show that in exponentially growing Chinese hamster ovary cells the five main histones are phosphorylated, and that, in each species, the variants are differently modified.

MATERIALS AND METHODS

The culture of CHO cells in monolayers has been previously described (9). Prior to labelling, cells were incubated for 1 h in Eagle's minimum essential medium, without phosphate, supplemented with 1/100 vol. complete medium 0111. 5 x 10^7 cells were labelled for 4 h in the same medium containing (^{32}P) orthophosphate (40 µCi/ml; 50 Ci/mg, Amersham England). After labelling cells were harvested and nuclei prepared according to Zalta et al. (10). Proteolytic inhibitors (1 mM phenylmethylsulfonyl fluoride and 1 mM diisopropylfluorophosphate) and an inhibitor of dephosphorylation and proteolysis of histones (5 mM 5.5' dithio-bis-2 nitrobenzoïc acid) (II) were included in all buffers. During the fractionation all the histones remain in the nuclear fraction. Acidic extraction of histones was carried out as described by Jackson (12).

Histones were analysed by slab gel electrophoresis as previously described (13), except that acrylamide gradient concentration was between 15 and 22 % to get a better resolution of different histones. After 15 h electrophoresis at 25 mA, the gel was rapidly stained with coomassie blue and bands corresponding to histones were cut out, put in a dialysis bag and submitted to an electroelution for 15 h at 40 mV, after immersion into Laemlli buffer (14), added with 2 % β mercaptoethanol. Samples were then precipitated with 25 % trichloroacetic acid and after dissolution analysed on a Triton acid urea slab gel (6 mM Triton, 7.5 M urea) (3). After coloration with coomassie blue or amido black, the gels are treated for 10 min. at 100°C with 15 % trichloroacetic acid, then washed for 1 h several time with 5 % trichloroacetic acid to remove nucleic acids. Gels were dried and exposed for autoradiography to Kodirex autoprocess (2 days for H2A to 20 days for H2B).

RESULTS

Acidic extraction of nuclei or chromatin results in the solubilization of the major part of histones (70 to 80 %). However when the unextracted material is solubilized in sodium dodecyl sulfate and analysed by gel electrophoresis, it becomes apparent that each histone species as was previously pointed out by Bonner et al. (15) for H4, are unequally solubilized. As shown in table 1, more than 90 % of H1, H2A and H2B are extracted by the acidic treatment of nuclei while almost 50 % of H3 and H4 remains in the acid insoluble fraction. The modified histones (phosphorylated and acetylated forms) are also not randomly distributed between these two fractions (results not shown).

TABLE 1 : Acidic extractibility of histones - level of phosphorylation.

	H1	H2A	H2B	H3	H4
Solubilized (%)	95 ± 10	90 ± 10	80 ± 10	52 ± 8	40 ± 8
SA Hx/SA H2A	0.83 ± .1	1	0.02 ± .01	0.14 ±.01	0.09 ±.01

Nuclei were extracted by acid (12). The unsolubilized fraction was dissolved in SDS buffer and analysed in parallel with the solubilized material by SDS slab gel electrophoresis. After staining gels were scanned and percentage of histone in each fraction determined. SA : specific activity of histones determined after scanning of the Triton-acidic-urea stained gels and of the autoradiograms (fig. 1).

Thus, to compare the level of phosphorylation of the different histones and of their variants in CHO cells, we have developed an experimental procedure which allowed firstly a complete extraction and recovery of the five major histones and secondly an excellent separation of the different variants. As shown in fig. 1, the five histones are well resolved by sodium dodecyl sulfate slab gel electrophoresis and phosphorylated histones are visualized by autoradiography of the gels. H2A and H1 are the most phosphorylated histones and present a specific activity six times higher than H3 and H4 while H2B is only slightly labelled. In all these experiments, nucleic acids have been removed by boiling of the gels in 15 % trichloroacetic acid before the autoradiography. The presence of label in these species could result from a true biochemical modification of the protein or from the presence of a phosphorylated contaminating protein that comigrates with the histone.

To discriminate between these two hypothesis, the bands containing individual histones are cut out of the gels and histones are recovered by electroelution with a yield around 100 %. The specific activity of each individual protein was identical before and after electroelution.

Fractions containing each major type of histones are analysed by Triton-acidic-urea gels in which the different variants are clearly separated. Phosphorylated species were then detected by autoradiography

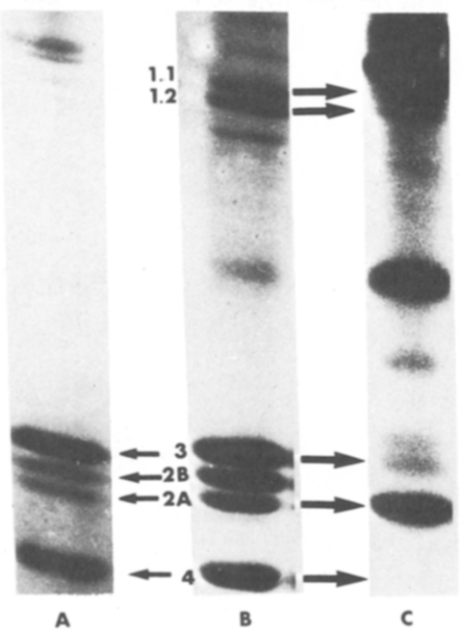

FIGURE 1 : Nuclei of ^{32}P labelled cells were solubilized in SDS buffer and proteins were analysed by slab gel electrophoresis (B, C). B : coomassie blue staining. C : autoradiograms of dried gels. A : the unsolubilized fraction of acid nuclear extract was dissolved in SDS buffer and analysed in parallel.

(fig. 2). Histone H4 is resolved into two main bands that are present in equal amount and that correspond to the unacetylated and the mono-acetylated forms (fig. 2c). The di- and tri-acetylated forms are also detected when deacetylase inhibitors are added during the fractionation. These species were characterized by running in parallel H4 extracted from cells treated with 5 mM sodium butyrate during 24 h (16) and in which all the acetylated forms (1 to 4) are equally present (fig. 2e). As shown in fig. 2d, two phosphorylated bands are detected between the mono- and di-acetylated H4 and around the diacetylated form. Systematically, the auto-radiogram indicates that the phosphorylated forms of histones migrate more slowly than the unphosphorylated forms as firstly described by Chalkley (17) (fig. 2a-2b). Approximatively, an equal proportion of non-acetylated and mono acetylated form is phosphorylated (less than 5 %). An additional

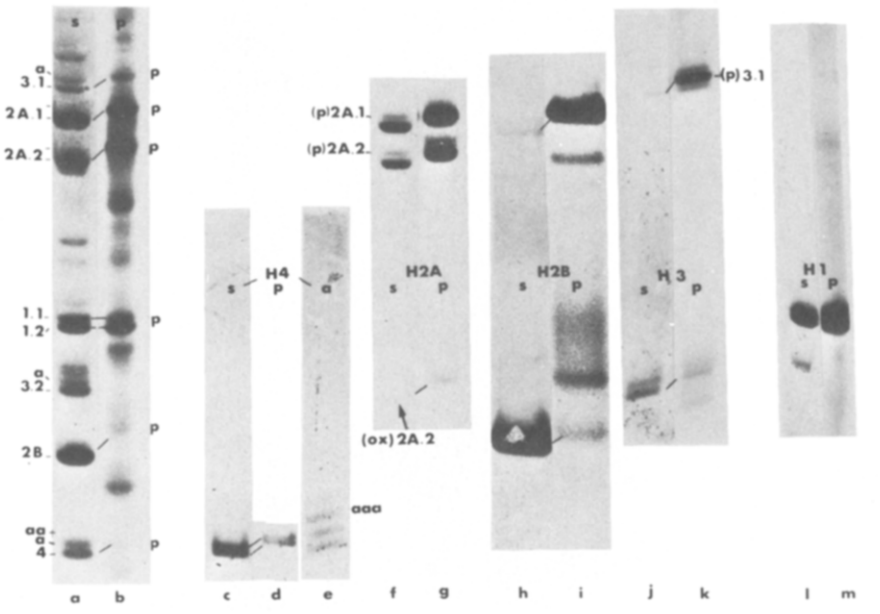

FIGURE 2 : (a, b) : total acidic nuclear extract was analysed by electrophoresis on Triton-acidic-urea gels. s : coomassie blue staining. p : autoradiogram. a : nuclear extract from butyrate treated cells. As phosphorylated H1 comigrates with the stained band, H1 are added in each gel analysis and are used as marker for both staining and autoradiogram (c to m).

band, that could correspond to the phosphorylation of the diacetylated form was also visualized after long exposure time of the autoradiogram. Histone H2A is fractionated into the two major variants H2A1 and H2A2 that are present almost in equal amount in the nuclei of CHO cells. In addition three minor variants are detected two of them migrating respectively just slower than H2A1 and H2A2 and another one representing 10 % of total H2A (fig. 2 f). It must be noted that the two variants H2AX and H2AZ recently described by West and Bonner (6), as ubiquitine-H2A complexes are not present since they possess molecular weight different from the major H2A and as such they do not comigrate in the preparative sodium dodecyl sulfate gel. As shown in fig. 2g, the two variants that migrate slower than H2A1 and H2A2 are the phosphorylated forms of these two proteins and represent respectively 30 % and 26 % of each variant. A third phosphorylated

band was observed just above the oxydised form of H2A2 and could be its phosphorylated form and represent 20 % of this species.

Histone H2B is resolved in two main bands (50 % each) that cor--respond to variants H2B1 and H2B2 (18). In this fraction is also present in low amount (less than 5 %) a protein which migrates as histone H2A1 (fig. 2h). On the autoradiogram is detected a band which migrates just slower than H2B2 and corresponds to its phosphorylated form. It represent less than 2 % of the unphosphorylated H2B. The main band corresponds to the phosphorylated form of H2A1 that contaminates this fraction; a low amount of the phosphorylated H2A2 is also present. This result shows an unequal contamination of H2B by H2A1 and H2A2 in sodium dodecyl sulfate gels as the presence H2AX which is approximatively 1000 daltons larger than H2A1 (6) and whose phosphorylated form is detected above the phospho rylated H2A1 (fig. 2i).

The H3 fraction is resolved by electrophoresis on acid Triton-urea gels in six variants which are respectively H31, H32 and its two acetylated forms, H33 and the monoacetylated H33 (fig. 2j). On the autoradiogram, a major band is detected which corresponds to the phosphorylated form of H31 while the additional minor band could correspond to the phosphorylation of the mono-acetylated form of H31 which is not detected by staining of the gel. The other bands are poorly labelled and correspond to the various forms of H32 and H33 described above (fig. 2k).

Histone H1 is fractionated into two main bands in the ratio two third, one third (fig. 2l). The analysis of the autoradiogram shows a complete correspondance between the stained bands and the phosphorylated bands which means that all the H1 molecules are phosphorylated (fig. 2m). The specific activity of H12 is two times higher than the specific activity of H11 which is in agreement with the results of Ajiro et al. (7).

The main following conclusions are drawn : 1 - the analytical system used allowed the study of all histone variants without any quali-

tative or quantitative loss. 2 - all the histone variants are phosphorylated in unsynchronized CHO cells. It has been reported that H2AZ is unphosphorylated (6). This species has not been analysed in this work since it migrates faster than H2A in sodium dodecyl sulfate gel electrophoresis (6) and, thus, was not cut out of the preparative gel. Similarly, the ubiquitine-H2A (6) and ubiquitine-H2B (18) were not analysed. 3 - all the detected acetylated forms of histones are also phosphorylated. 4 - the specific activity of the phosphorylated histones is markedly different after a 4 h *in vivo* labelling with ^{32}P orthophosphate (from 1 for H2A to 0.1 for H2B). Since a maximum of three addition sites is present by molecule, this result suggest that the turn over rate of phosphate groups of various histones is different what was confirmed by an independent approach (manuscript in preparation).

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