DNA-BOUND HISTONES ARE NOT PHOSPHORYLATED BY PROTEIN KINASES

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1. Introduction

Histones are phosphorylated in proliferating cells with increasing rates at the beginning and during the DNA replication phase of the cell cycle (reviewed [1,2]). It appears that newly synthesized histones are preferentially phosphorylated [3,4], and it has been speculated that this type of post-translational modification is required for the proper assembly of chromatin.

We have previously observed that no phosphorylation sites are available for nuclear protein kinases in DNA-bound histone H1 and in H1 in isolated (presumably) intact chromatin [5]. Free histone H1, on the other hand, is an excellent substrate for the major nuclear and cytoplasmic protein kinases.

In this communication we show that the other histone species behave in a similar way: histones H2a, H3 and H4 (but not H2b) are readily phosphorylated by two major mammalian protein kinases when free in solution; the same histone preparation is not phosphorylated when bound to DNA. These observations provide further evidence suggesting that most histone phosphorylation may occur prior to chromatin assembly.

2. Materials and methods

2.1. *Preparation of histones*

Histones were prepared from chromatin of non-proliferating mouse ascites cells by 2 M NaCl and 5 M urea as in [5]. Histone H1 was separated from the other four histone species by chromatography on CM cellulose columns [6]. The histones H2a, H2b, H3 and H4 were precipitated by 8 vol. acetone at −20°C and resuspended at a concentration of about 1 mg/ml in 2 M NaCl, 20 mM Na_2HSO_3 and 50 mM Tris–HCl, pH 7.5.

2.2. *Protein kinases*

Protein kinases were prepared from concanavalin A activated bovine lymphocytes [7].

The soluble cAMP-dependent enzymes were purified about 300-fold from a 100 000 × *g* supernatant of a cell extract as in [8]. The protein kinase activity of peak I as defined [8] was used for the experiments below.

The major chromatin-bound cAMP-independent protein kinase was prepared as in [8]. This activity was purified about 500-fold from a 0.4 M NaCl extract of isolated lymphocyte chromatin.

The activity of the protein kinases was determined at pH 7.0 in a phosphorylation buffer containing 25 mM sodium phosphate, 10 mM $MgCl_2$, 15 mM 2-mercaptoethanol and 0.1 mM [γ-^{32}P]ATP (spec.act. 1000–2000 cpm/pmol) (Amersham-Buchler). The ^{32}P transferred to histones was determined in an acid precipitate which was prepared for counting as in [5]. One enzyme unit transfers 1 pmol phosphate/min at 37°C to acid-precipitable protein [8].

2.3. *Binding of histone to DNA*

To prevent non-specific aggregation of histones and DNA, a modification of the gradient dialysis procedure [9] was used. DNA (to be specified below) and histones H2a, H2b, H3 and H4 were mixed in 2 M NaCl, 5 M urea, 20 mM NaH_2SO_3, 30 mM 2-mercaptoethanol and 100 mM Tris–HCl, pH 7.5. The concentration of Na_2HSO_3, Tris–HCl and 2-mercaptoethanol remained constant throughout the experiment while the concentration of NaCl

and urea was gradually reduced. For this purpose, 2 ml samples of the histone–DNA mixture were first dialysed for 2–3 h against 100 vol. starting buffer, then for 6 h against 1 M NaCl (no urea), for 12 h against 0.8 M NaCl, for 6 h against 0.6 M NaCl and for 12 h against 0.4 M NaCl.

2.4. *DNA*

Bacteriophage T7 DNA was prepared from 10^{12}–10^{13} phage particles in 5 ml Tris–HCl buffer (50 mM Tris–HCl, pH 7.5; 5 mM EDTA). To the phage suspension were added SDS (final conc. 0.5%) and 2-mercaptoethanol (final conc. 1%). After 60 min at 45°C, the DNA was extracted 3 times by buffer-saturated phenol at 45°C. The aqueous phase was then dialysed against Tris–EDTA buffer to remove the remaining phenol. The DNA was then precipitated with ethanol and resuspended in Tris–EDTA buffer to give conc. 400–500 μg/ml.

Col E1 [^{3}H]DNA was prepared from *Escherichia coli* cells as in [10].

DNA concentrations were determined using $E^{1\,cm}_{260\,nm}$ = 20 for 1 mg/ml DNA.

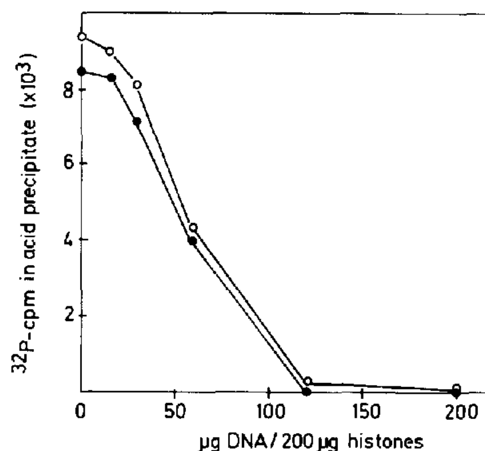

Fig.1. Phosphorylation of DNA-bound histones? Six samples containing 200 μg histones were mixed with increasing amounts of bacteriophage T7 DNA. The volumes were adjusted to 2 ml. Enough NaCl and urea were added to give the required initial concentrations of 2 M and 5 M, respectively. Gradient dialysis was performed as described. After dialysis, samples were diluted 1:10 in phosphorylation buffer, containing 5 × 10^{-6} M cAMP. The reactions were started by addition of about 1 unit each of the cAMP-dependent cytoplasmic protein kinase (•) and the cAMP-independent chromatin-bound protein kinase (○).

3. Results and discussion

Bacteriophage T7 DNA (mol. wt 25 × 10^6) [11] and a histone preparation, containing H2a, H2b, H3 and H4, were mixed in different weight ratios as indicated in fig.1. The mixtures were then used for gradient dialysis as above. (It should be noted that according to [9] no special base sequence is required for nucleosome formation.) After gradient dialysis, a 0.025 ml sample from each mixture was added to 0.225 ml phosphorylation buffer. The reaction was started by addition of the cAMP-dependent cytoplasmic protein kinase and, in a parallel experiment, by addition of the cAMP-independent major chromatin-bound protein kinase. The results, as shown in fig.1, demonstrate that, with increasing DNA/histone ratio less ^{32}P appears in the acid precipitate. Virtually no ^{32}P was detected in acid precipitable material at a DNA/histone ratio of 0.5 and above regardless of whether the cytoplasmic enzyme or the chromatin-bound protein kinase was used for the reaction. At low DNA/histone ratios some degree of phosphorylation is observed. This is most probably due to the presence of unbound histones. It is also possible, however, that under the experimental conditions some histones are bound to DNA, but in a way which leaves some or most phosphorylation sites open for phosphate transfer. The latter possibility was investigated. Col E1 [^{3}H]DNA was added to ascites cell histones in a DNA/histone ratio of 0.1. After gradient dialysis, the complexes were sedimented through sucrose gradients containing 0.4 M NaCl. Protein determination in each fraction of the gradient showed that about 20% of all recovered histone protein was associated with the fast-sedimenting complex; the remainder was found close to the top of the gradient (fig.2). This observation is in agreement with [13], that mammalian histones bind in a regular fashion to Col E1 DNA. Complexes were found with a DNA/histone (w/w) ratio of maximally 0.33 even when a high excess of histone was added to the DNA before gradient dialysis [13].

Samples containing equal amounts of protein were then removed from the gradient of fig.2 and added to phosphorylation buffer containing protein kinase.

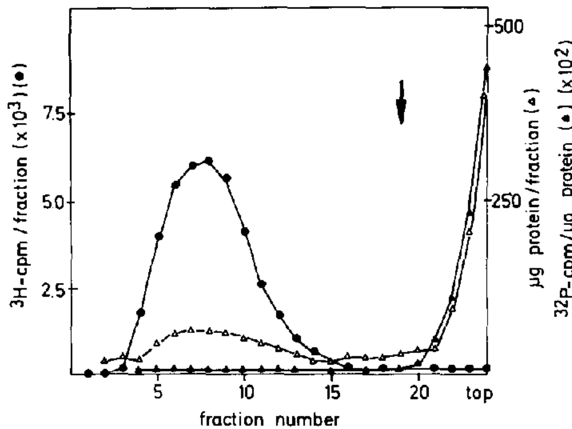

Fig.2. Histones bound to Col E1 DNA. Col E1 [^{3}H]DNA (41 500 cpm) 100 μg were mixed with 980 μg histones in 1 ml. After gradient dialysis, the mixture was sedimented through a 16 ml sucrose gradient (20–5% sucrose in 0.4 M NaCl; 50 mM Tris–HCl, pH 7.5; 1 mM EDTA; 15 mM 2-mercaptoethanol). For centrifugation, the SW27 rotor was used at 26 000 rev./min, 20°C, for 2.5 h. ^{3}H cpm were determined in 0.1 ml samples taken from the fractions of the gradient (●). The protein concentrations in 0.1–0.2 ml samples from each fraction were determined as in [12] (▵). Samples containing 2 μg protein were then taken from each fraction and diluted 1:5 in phosphorylation buffer. Chromatin-bound protein kinase (1 unit/ml) was added to start the reaction which was performed at 37°C for 40 min (▴). An untreated Col E1 DNA was centrifuged under identical condition in a parallel centrifuge tube. The position of the peak fraction of uncomplexed DNA in the gradient is indicated by the arrow.

The results, as presented in fig.2, show that histones bound to the plasmid DNA are not phosphorylated while free histones are good substrates for the protein kinase.

It is further necessary to show that DNA does not directly affect the protein kinase activity. This was excluded by the observation that casein is equally well phosphorylated in the absence and in the presence of a 10-fold excess of T7 DNA (data not shown).

We have also investigated whether phosphorylation affects the binding of histones to DNA. For this purpose, about 100 μg of histone preparations, H2a, H2b, H3 and H4, were incubated with protein kinase in phosphorylation buffer. Separation of the histones by gel electrophoresis followed by autoradiography (fig.3) showed that all histones, with H2b excepted, were phosphorylated. Crude estimates based on the concentration of histones and the specific activity of the [γ-^{32}P]ATP used showed that, on the average, 1–2 mol phosphate were transferred to histones H2a, H3 and H4. About 20 μg modified histone preparation was mixed with 10 μg Col E1 DNA. A control contained identical concentrations of unmodified histones and DNA. After gradient dialysis, the com-

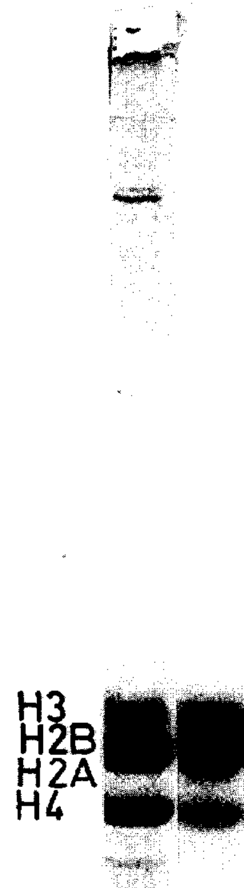

Fig.3. Gel electrophoresis of phosphorylated histones. About 100 μg histones in 2.5 ml phosphorylation buffer (containing 0.5 mM [γ-^{32}P]ATP were incubated for 2 h with 20 units chromatin-bound protein kinase at 37°C. At this time, more enzyme was added and the incubation continued for another 1 h. The histones were then precipitated by acetone (see above) and resuspended in the 2 M NaCl–5 M urea buffer for gradient dialysis. A 0.02 ml sample was removed and investigated in the SDS–gel electrophoresis system [14] using a 15% polyacrylamide slab gel. After electrophoresis and staining, the gel was used for autoradiography. Left: Stained gel. Right: Autoradiogram of the same gel.

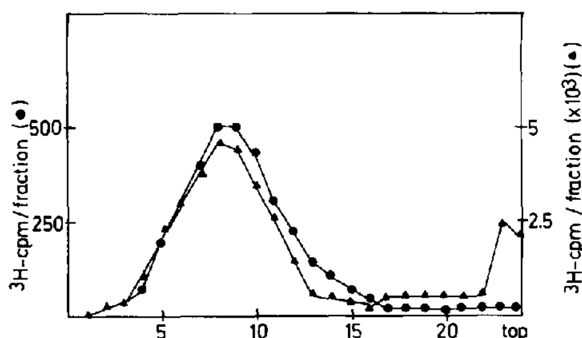

Fig.4. Binding of phosphorylated histones to DNA. A sample containing 20 μg phosphorylated histones from the preparation in fig.3 was mixed with 10 μg Col E1 [^{3}H]DNA. In this experiment, 0.5 μg/ml bovine serum albumin was added to the mixture before gradient dialysis was started. In a parallel dialysis bag, unmodified histones and Col E1 DNA were used for reconstitution under identical conditions. The DNA histone complexes were centrifuged as in fig.2. At the end of the centrifuge run, fractions were collected on glass fiber filters. The samples were processed for radioactivity counting as in [5]. ^{3}H counts (●) indicate the position of the DNA; ^{32}P counts (▲) represent the phosphorylated histones. We present only the result of the experiment with phosphorylated histones since the histone–DNA complex in the control experiment sedimented exactly like the one shown above (fig.2)

plexes were sedimented through sucrose gradients as in fig.2. We found (fig.4) that the complexes had very similar sedimentation properties regardless of whether phosphorylated or unmodified histones had been used for the experiment.

The resolution of this type of experiment is, of course, too crude to allow the detection of changes in the quarternary structure of nucleosomes. It shows, however, that phosphorylated nucleosomal histones do bind to DNA and that the gross conformation of this DNA–histone complex is similar to a complex with unmodified histones.

Acknowledgement

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