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Histone acetylation and gene induction in human cells

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An antibody recognising acetylated core histones was used to immunoprecipitate chromatin fragments from proliferating human K562 cells and from cells induced to differentiate with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA). The DNA of the acetylated chromatin was probed with sequences of platelet derived growth factor B chain (PDGF-B), a gene which is induced to strong expression upon differentiation. A high level of acetylation was observed before gene induction and no change seen following induction. This implies that core histone acetylation is an essential precondition for transcription.

Histone acetylation; Active chromatin; Induction

1. INTRODUCTION

The post-translational acetylation of specific lysine residues in the N-terminal domains of core histones has been biochemically linked to transcriptionally active chromatin by immunoprecipitation with an antibody that specifically recognises all acetylated core histones. Probing the DNA of such chromatin from 15-day chicken embryo erythrocytes with sequences of a gene active in that tissue (α^D globin) showed high enrichments. Probing with sequences of the inactive gene, ovalbumin, showed no enrichment [1]. That inactive genes do not carry acetylated histones has been confirmed for H4 in particular by the demonstration that the human female inactive X chromosome fails to stain with antibodies to acetylated H4, in contrast to all the other chromosomes [2]. The importance of acetylation for transcriptional control has also been shown by genetic experiments with yeast in which mutation of the critical lysine residues in H4 led to repression of the GAL1 and PHO5 genes [3]. The role of core histone acetylation in transcription has been reviewed [4,5]. At the chicken β globin locus a switch occurs between transcription of the embryonic ρ gene, which is active in the 5-day embryo and the adult β gene, which is active in the 15-day embryo. Immunoprecipitation of chromatin fragments from each stage with an antibody to acetylated histone demonstrated enrichment of sequences from both the active and inactive globin genes at each stage [6]. This result implied that a 'poised' globin gene carries acetylated histones like an active globin gene. 5-day chicken erythrocytes represent the primitive cell line in which the adult gene will never be transcribed and conversely in the definitive cell line at 15-days the embryonic gene has never been transcribed. The globin switch does not therefore occur within individual cells. The experiments reported here address the question of the acetylation status of a gene that can be induced within a single cell type and can thus be regarded as 'poised' in the uninduced cell. We have looked at the induction of the PDGF-B gene in K562 cells to ask if induction of a gene can give rise to increased levels of acetylation on the histones of its chromatin.

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2. MATERIALS AND METHODS

2.1. *Antibodies*

Affinity-purified polyclonal antibodies which recognise the epitope ε-acetyl lysine and therefore all modified core histones were prepared as described previously [7].

2.2. *Cell culture*

K562 cells were grown at 37°C, 5% CO_2, in RPMI 1640 medium, containing 2g/l sodium bicarbonate, supplemented with 10% heat inactivated newborn calf serum, 2 mM L-glutamine, 50 U/ml streptomycin/50 μg/ml penicillin and non-essential amino acids (NEAA). Large scale cultures in 5l spinner flasks were maintained at a density between 5×10^5 and 10^6 cells/ml by adding fresh medium daily. For TPA treatment, cells were pelleted at $225 \times g$ for 10 min at room temperature and resuspended in the same volume of medium containing 10^{-9}M TPA (Sigma). Cells were incubated in the presence of TPA for 48 h, harvested by centrifugation at 4°C at $2,000 \times g$ for 20 min) washed in phosphate-buffered saline (PBS),10 mM sodium butyrate and re-centrifuged as above.

2.3. *Preparation of nuclei*

K562 cells were lysed in 250 mM sucrose, 10 mM Tris-HCl, pH 7.4, 10 mM sodium butyrate, 4 mM $MgCl_2$, 0.1 mM PMSF, 0.1 mM benzamidine, 0.1% Triton X-100, using 10 strokes of a Dounce homogeniser. Nuclei were prepared for micrococcal nuclease digestion as in [6] modifying the digestion buffer to include 250 mM sucrose.

2.4. *Preparation of chromatin*

Nuclei were digested at a DNA concentration of 5 mg/ml with 80 U/ml micrococcal nuclease for 10 min at 37°C. Digestion was termi-

nated by the addition of Na_3EDTA to a final concentration of 5 mM. The suspension was centrifuged at 800 × *g* for 15 min and the supernatant (S1) retained. The nuclear pellet was resuspended in 10 mM Tris-HCl pH 7.5, 10 mM sodium butyrate, 0.25 mM Na_3EDTA, 0.1 mM PMSF, 0.1 mM benzamidine (lysis buffer), left on ice to lyse for 15–20 min and re-centrifuged as above, retaining supernatant (S2). Supernatants S1 and S2 were pooled, 5 M NaCl added to a final concentration of 150 mM and samples chilled on ice for 20 min to precipitate H1 containing chromatin, which was removed by centrifugation at 8000 × *g* for 20 min at 4°C. The NaCl concentration was reduced to 50 mM and residual H1 removed by the addition of 30 mg/ml Sephadex CM 25 for 1 h at 4°C with constant mixing. 'Salt soluble' chromatin fragments prepared by these digest conditions are mainly mono and dinucleosomes with some tri and tetra nucleosomal fragments. Mononucleosomes were purified by centrifugation of H1 depleted chromatin for 17 h at 40,000 r.p.m., 4°C in a Beckman SW40 rotor, through 5–30% exponential sucrose gradients containing 10 mM Tris-HCl pH 7.5, 10 mM sodium butyrate, 0.25 mM Na_3EDTA, 50 mM NaCl, 0.1 mM PMSF, 0.1 mM benzamidine. Chromatin fragments were then dialysed into 10 mM Tris-HCl pH 7.5, 10 mM sodium butyrate, 50 mM NaCl, 1 mM Na_3EDTA, 0.1 mM PMSF, 0.1 mM benzamidine (incubation buffer) ready for immunoprecipitation.

2.5. *Immunoprecipitation of chromatin*

This was performed as described previously [1,6] by mixing salt soluble chromatin containing 400 μg DNA with 100 μg of antibody in 750 μl of incubation buffer for 2 h at 4°C. Immunocomplexes were precipitated with formalin-fixed *S. aureus* cells (Immunoprecipitin, GIBCO). Chromatin and antibody were released from the *S. aureus* cells in buffer containing 1.5% SDS and DNA obtained by phenol/chloroform extraction. The DNA was treated with RNase A and proteinase K before dotting. Proteins were isolated from the phenol/chloroform phase by the addition of HCl and precipitated with acetone.

2.6. *RNA preparation and Northern analysis*

Total cellular RNA was isolated by extraction with 4 M guanidinium isothiocyanate and caesium chloride centrifugation as described in [8]. 30 μg RNA was electrophoresed through a 1.4% agarose, 2.2 M formaldehyde gel essentially as in [9] and then transferred to Biodyne B (Pall) nylon membranes in 20 × SSC. Membranes were rinsed in 2 × SSC, blotted dry and the RNA crosslinked by UV irradiation.

2.7. *Slot blots*

DNA samples, quantified by UV absorption, were denatured in 500 mM NaOH, 1.5 M NaCl for 10 min at 37°C and 1 min at 100°C, then applied to Biodyne B filters (Pall). The filters were washed in 0.5 M Tris-HCl pH 7.2, 1.5 M NaCl, 1 mM Na_3EDTA for 30 s before blotting dry and fixed by baking at 80°C for 30 min.

2.8. *Hybridisation*

Filters were probed with either a 1.5 kb genomic fragment containing the whole of the human α globin gene, a 300 bp PDGF-B cDNA fragment, a 1.2 kb glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA or a 600 bp human growth hormone (HGH) cDNA fragment, provided by the American Type Culture Collection (A.T.C.C), labelled with ^{32}P by random priming. Hybridisation was carried out at 68°C using Quickhyb hybridisation solution (Stratagene). Following hybridisation the final wash was in 0.2 × SSC, 0.1% SDS for 15 min at 65°C. Filters were blotted dry and autoradiographed.

2.9. *Electrophoresis*

Proteins from chromatin fractions were analysed on 15% polyacrylamide gels containing 0.9 M acetic acid, 6.25 M urea as described in [10] and stained with 0.1% Coomassie R250.

3. RESULTS AND DISCUSSION

The human haematopoietic stem cell line K562 can be regarded as erythroid since the α globin gene is actively transcribed. The platelet derived growth factor B chain (PDGF-B) gene is reported to be inactive in growing K562 cells but induced when cells are treated with the phorbol ester TPA to cause differentiation along the megakaryocyte lineage [11]. Using the antibody that recognises all acetylated core histones [1], salt soluble chromatin fragments from both uninduced and TPA treated K562 cells were immuno-precipitated and their DNA probed with several gene sequences.

3.1. *The proteins of antibody fractionated K562 chromatin*

Fig. 1a shows an acetic acid/urea gel of histones from an immunoprecipitation of mononucleosomes from rapidly dividing K562 cells. The modest levels of acetylation in the input chromatin (**I**) (e.g. mono and non-acetylated H4) are also seen in the unbound (**U**) supernatant. This correspondance is expected since the unbound fraction represents ~95% of the input chromatin. In contrast, histones from the antibody bound (**B**) pellet fraction show the more highly acetylated forms of H4 and H3 and the H2A/2B region shows the multiplicity that arises from H2B acetylation. This demonstrates that the immunoprecipitation is able to select a highly acetylated population of nucleosomes. A similar pattern of core histone acetylation is seen in polyacrylamide gels of proteins extracted from salt soluble chromatin, but in this case overlaid with a number of other bands from proteins that are removed in the purification of mononucleosomes (data not shown).

3.2. *The DNA of antibody fractionated K562 chromatin*

Fig. 1b shows the results of hybridisation experiments on the DNA extracted from an immunoprecipitation of salt soluble chromatin fragments, using as probes the sequences of an actively transcribing gene (α globin), a silent gene (human growth hormone, HGH) and an inducible gene (PDGF-B). Equal amounts of DNA (2μg) from **I**, **U** and **B** were dotted onto the filters. As expected from previous results on the active chicken α^D and β globin genes [1,6], there is strong enrichment of the tissue specific human α globin sequences in **B** relative to **I**, and no enrichment of HGH sequences. However, sequences of the inducible PDGF-B gene are also enriched in the antibody bound chromatin. Since the PDGF-B gene is reported to be non-transcribing in proliferating K562 cells [11], the observation that it carries acetylated histones parallels the previous finding that 'poised' globin genes in chicken erythrocytes are modified. Densitometry of the autoradiographs shows that the level of enrichment of PDGF-B sequences in the antibody bound chromatin is somewhat less than for the tissue specific α globin gene and so we wished to check

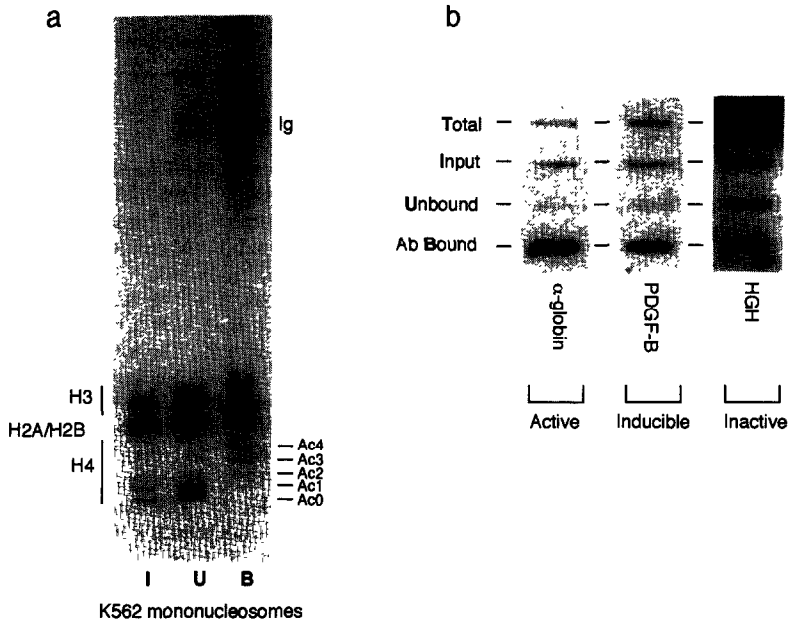

Fig. 1 (a) Acetic acid/urea polyacrylamide gel of core histones extracted from mononucleosomes. The three tracks show proteins from input (**I**), the unbound supernatant (**U**) and the antibody bound pellet (**B**). (b) Slot blot hybridisation of DNA extracted from **I**, **U** and **B** salt soluble chromatins with three different gene sequences. **T** = total genomic DNA. 2μg DNA loaded in each slot.

whether induction of the PDGF-B gene results in an increase in histone acetylation.

When K562 cells are induced to differentiate, mRNA transcripts of the PDGF-B gene are reported to become detectable 4 h after TPA treatment, with substantial amounts detected after 2 days [11]. K562 cells were therefore treated with 10^{-9} M TPA for 48 hr and then harvested. Fig. 2a shows a Northern analysis of total cellular RNA from treated and proliferating cells, probed with PDGF-B sequences and then subsequently probed with sequences of GAPDH to monitor for constant RNA loadings. The results demonstrate that only a very low level of PDGF-B transcripts are present in proliferating K562 cells but TPA treatment induces a considerable increase. Since nuclear run-off assays have also shown a sharp increase in transcription following TPA treatment [11], it is clear that the rise in mRNA level is due to the up-regulation of transcription rather than the stabilisation of pre-existing transcripts.

Salt-soluble chromatin was prepared from both proliferating and TPA treated K562 cells and immunoprecipitated. DNA from the various fractions was extracted and equal amounts dotted as before (Fig. 2b). Probing with PDGF-B shows that the gene is enriched in **B** prior to stimulation (as also shown in Fig. 1b) but there is no increase in enrichment after stimulation. As a control, an identical filter was probed with HGH sequences and no enrichment was seen. Both filters

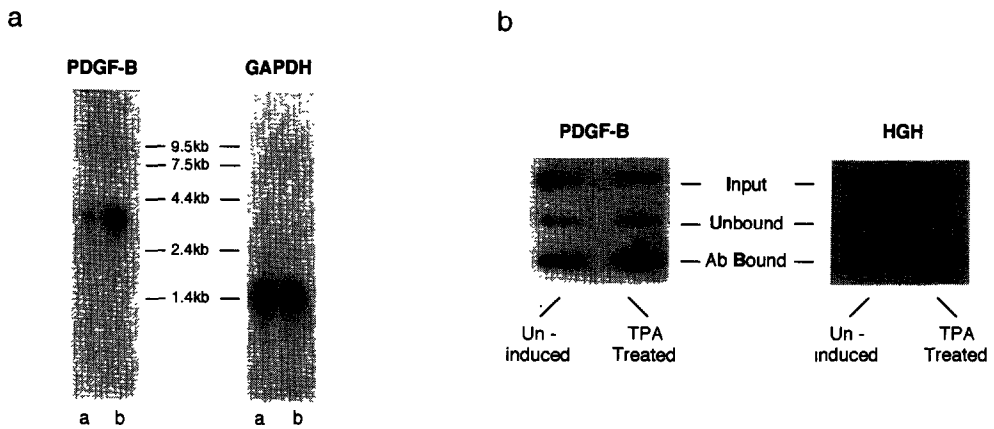

Fig. 2 (a) Northern analysis of total cellular RNA (30 μg per track) Lanes a: RNA from proliferating K562 cells. Lanes b: RNA from TPA treated, differentiating K562 cells. (b) Hybridisation of DNA extracted from **I**, **U** and **B** salt soluble chromatins with PDGF-B and HGH sequences. 2 μg DNA per slot.

shown in Fig. 2b were then stripped and re-probed with α globin sequences: enrichments similar to those seen in Fig. 1b were observed (data not shown).

We conclude that in human cells, not only does an actively transcribing gene (α globin) carry acetylated histones but an inducible gene (PDGF-B) also carries the modification prior to induction and the level of histone acetylation does not change when the gene is significantly up-regulated following induction by an external stimulus. It is interesting to compare the present results with those from experiments describing the fractionation of unfolded nucleosomes using mercury affinity columns. The unfolded nucleosomes retained were shown to be enriched in both transcriptionally active genes and highly acetylated histones [12]. In further experiments with inducible genes of mouse 3T3 cells the authors observed retention of c-myc and c-fos nucleosomes only when the genes were actively transcribing, but not in the 'poised' or 'switched-off' state [13]. Since the present data show that the inducible PDGF-B gene is acetylated prior to the onset of transcription, the two sets of observations taken together show that acetylation alone does not give rise to the unfolded state, which must be generated by additional factors. A high level of histone acetylation therefore represents the 'ground state' of all 'poised' genes.

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