Nucleosomal structure at hyperacetylated loci probed in nuclei by DNA–histone crosslinking

Konstantin K. Ebralidse†, Tim R. Hebbes, Alison L. Clayton, Alan W. Thorne and Colyn Crane-Robinson*

Biophysics Laboratories, University of Portsmouth, St Michael’s Building, White Swan Road, Portsmouth PO1 2DT, UK

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ABSTRACT

Chemically induced histone-DNA crosslinking in nuclei is used to monitor structural changes in chromosomal domains containing hyperacetylated histones. Core particles harbouring the crosslinks are immuno-fractionated with antibodies specific for acetylated histones. Crosslinking is revealed by gel separation of tryptic peptides from core histones that carry 32P-labelled residual nucleotide. The large number of DNA-histone crosslinks retained indicates that acetylated core histone tails are not totally displaced from the DNA. Changes in the patterns of crosslinked peptides imply a restructuring of hyperacetylated histone-DNA interactions at several points within the nucleosome. This demonstrates that a distinct conformational state is adopted in acetylated nucleosomes, known to be concentrated at transcriptionally active loci.

INTRODUCTION

The details of the chromatin structure of transcriptionally active genes is a major unresolved problem of eukaryotic gene expression. Nucleosomes, or nucleosome-like structures, are present on the protein-coding genes transcribed by RNA polymerase II (reviewed in [1]). Chemical crosslinking has given direct evidence for the presence of histones in active chromatin but indicated a different mode of binding from that in inactive chromatin [2].

Although it is possible that loss of the 30nm fibril structure is alone sufficient to render a locus transcribable, the observation of a number of biochemical changes suggests that several other preconditions for transcription must be met. Promoter regions have frequently been found to lack nucleosomes [2,3] and the presence of nucleosomes close to transcriptional start sites seems to prevent initiation. Elongation however is probably not prevented by the presence of nucleosomes [for reviews see 1,3,4]. The presence of acetylated histones at active and poised loci has been directly demonstrated by the use of an antibody that recognises the modified histones [5,6]. Physical studies demonstrate that hyperacetylation does not directly lead to gross structural changes either at the level of the 30nm fibril or at the level of nucleosomes; rather, there are subtle changes in the conformation of modified nucleosomes [7,8,9; see also 10 for a review]. It is not clear however whether nucleosomes located on the bulk of the transcribed regions of protein coding genes contain the usual octamer of histones in a ‘canonical’ core particle together with a linker histone molecule bound peripherally, or whether a new conformation is adopted to facilitate transcription.

This study uses crosslinking to look for changed nucleosomal structure in transcriptionally active and poised genes. This requires a protocol for the fractionation of active chromatin in a state pure enough to avoid gross dilution by inactive chromatin. To achieve this we used an antibody that recognizes acetylated core histones in an affinity purification of active chromatin. It has been shown that this antibody gives a 20—30 fold enrichment of active sequences for highly transcribed and also poised and previously active genes [5,6]. To analyse differences in the mode of core histone attachment to DNA in active versus inactive chromatin domains, we observe the regions of core histones which can be crosslinked to DNA within nuclei [2,11], and compare the crosslinking pattern from the immunoprecipitated fraction (taken as representing the active chromatin), with the pattern from the chromatin that fails to bind to the antibody (taken as representing the transcriptionally silent domains).

MATERIALS AND METHODS

Nuclei were isolated from 15-day chicken embryos by the method of Langmore and Paulson [12], with the addition of 10 mM sodium butyrate in all buffers.

DNA-histone crosslinking in nuclei

A) Pyridinium borane complex (PBC) reduction (LYS protocol). DNA was methylated by suspending nuclei on ice in 20 mM cacodylate-MMBI (modified magnesium-containing buffer [13]

* To whom correspondence should be addressed
† Present address: Harvard University, Department of Biochemistry and Molecular Biology, 7 Divinity Avenue, Cambridge, MA 02138-2092, USA and Permanent address: W. A. Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 32 Vavilov St., Moscow 117984, Russia.
with 10 mM sodium butyrate plus protease inhibitors diisopropyl fluorophosphate (DFP), phenylmethylsulphonyl fluoride (PMSF) and benzamidine hydrochloride (BA), 0.2 mM each) and adding dimethylsulphate (DMS) to 1 mM. The suspension was left for 12 hrs on ice and nuclei then resuspended in MMIB with PBC added to 8 mM. Depurination and reduction were effected by incubation at 37°C for 10 hrs. Reduction was completed by addition of sodium borohydride to 10 mM, on ice.

**B) Borohydride reduction (‘HIS’ protocol).** The same procedure as described above, except that no PBC was added.

**Preparation of nucleosomal core particles from cross-linked nuclei**

Nuclear pellets were suspended at 100 A260 U/ml in MMIB with 0.2 mM CaCl2 and digested with 5 μg/ml micrococcal nuclease (Sigma) at 37°C for 3 min. Following chilling and addition of EDTA, soluble chromatin at a final concentration of 33 A260 U/ml was obtained by dispersing the nuclei in NaCl/potassium phosphate buffer, pH 7.0 (0.6 M and 50 mM, respectively). The soluble chromatin was then rolled for 1 hr in the cold with 1/3 (bed) volume of the cation-exchange resin Dowex AG 50W X2 [14] equilibrated against the same buffer, centrifuged and dialyzed against HB buffer (25 mM HEPES-NaOH, pH 8.0, 10 mM sodium butyrate, 0.1 mM EDTA, 0.5 mM DFP, 0.5 mM PMSF, 0.5 mM BA). To obtain core particles, the second digestion with micrococcal nuclease, at 3 μg/ml, was performed at 37°C for 15 to 20 min. in the same buffer but with 0.5 mM CaCl2. The digest was dialyzed against NT buffer (50 mM NaCl, 10 mM Tris-Cl, pH 8.5, 10 mM Na butyrate, 1 mM EDTA, 0.5 mM DFP, 0.5 mM PMSF, 0.5 mM BA) and concentrated in a Centricon 30 microconcentrator (Amicon).

**DNA-histone crosslinking in nucleosomal core particles**

The DNA of nuclei was methylated and core particles prepared as above; the particles were dialyzed against HB buffer and then crosslinked by the above procedures. They were finally dialyzed against NT buffer.

**Immunofractionation of crosslinked chromatin**

0.6 ml of a 25 A260 U/ml crosslinked sample (i, 'input' chromatin) was mixed with 200 μl of 0.5 mg/ml affinity-purified antibody and rolled for 2 hrs at 20°C. Fixed S. aureus cells (Immuno precipitin, Gibco-BRL), washed exhaustively in NT, were then added and incubation continued for a further 1 hr at 20°C. After pelleting the cells, the supernatant was saved and the cells washed in NT buffer five times. The supernatants were combined and represented the 'unbound' (u) fraction. To release the antibody-bound fraction (b), the cell pellets were extracted twice with 1.5% SDS/NT buffer.

**Hydrolysis of DNA in the crosslinked chromatin fractions**

Samples were brought to 0.5% SDS/10 mM methylamine-HCl, boiled for 90 sec and immediately chilled on ice. KCl was added to 0.3 M, the samples were kept on ice for 30 min and pelleted. The pellets were washed with 80% ethanol, resuspended in 60 μl of 10 mM cysteamine-HCl, mixed with 120 μl of 3% diphenylamine in 98% formic acid, incubated at 70°C for 25 min, diluted with an equal volume of 10 mM cysteamine-HCl, extracted three times with ether and finally lyophylised. The dried residue was redissolved in 80 μl of a solution containing 0.05 μg/μl acid-extracted carrier histones, precipitated with 20 μl of 100% (w/v) trichloroacetic acid (TCA), washed twice with 0.02 M HCl in 90% acetone and dried under vacuum. The pellets were resuspended in 80 μl of 75 mM glycine-NaOH, pH 9.0, 1 mM TrisHCl, 1 mM CaCl2 with 0.05 μg/μl micrococcal nuclease and 0.01 μg/μl bacterial alkaline phosphatase (Sigma) and incubated at 37°C for 30 min. After EDTA addition and chilling on ice the proteins were precipitated with 20 μl 100% (w/v) TCA, washed with HCl/acetone and dried as above.

**Labelling the residual nucleotides linked to histones**

The proteins were suspended in 20 μl of 50 mM Glycine-NaOH, pH 9.0, 10 mM MgCl2, 10 mM DTT, 0.1 mM spermine, 0.2 mM PMSF and incubated with 5 U of polynucletide kinase at 37°C for 30 min. The reactions were stopped by EDTA addition and chilling on ice. TCA precipitation and HCl/acetone washes were as described above. Proteins were suspended in SDS-containing sample buffer and separated using 15% polyacrylamide discontinuous gel electrophoresis [13]. Wet gels were autoradiographed and the individual histone bands excised with a blade. Gel slices were extracted with 0.1% Triton X-100, 5 mM DTT at 45°C for 6 hrs. The eluates were lyophylised, washed with 2% LiClO4 in 85% acetone, then with HCl/acetone (as above) and dried.

**Trypsin digestion**

The dried pellets were dissolved in 20 μl of 0.1 M NH4HCO3, 5 mM DTT, 0.05 mM EDTA and incubated with TPCK-treated, HPLC-purified trypsin [11] at 50 μg/ml for 6 hrs at 37°C. The digests were lyophylised and resuspended in 5 μl of 5 mM DTT/bromophenol blue/xylene cyanol/10% Ficoll-400/15% (v/v) glycerol.

**Gel electrophoresis of nucleotide-peptide products**

18% polyacrylamide gels, 0.4×180 mm, contained 200 mM Glycine/25 mM Tris/0.1 mM EDTA (pH 8.2) buffer [11]. They were loaded with 2 μl of the protease-digested samples and run at 65 V/cm for 50 min, dried immediately after completion of electrophoresis and exposed to X-ray film without an intensifying screen.

**RESULTS**

**Design of the experiment**

Figure 1 gives a flow chart of the principal steps. The DNA in nuclei from 15-day chicken embryo erythrocytes was mildly methylated with dimethyl sulphate (at less than 1 methyl group per 200 bp) and the aldehyde groups generated by depurination at 37°C allowed to react with the ε-amino groups of lysine residues, the imidazoles of histidines and N-terminal α-amino or α-imino groups, wherever available. In the first reduction protocol, pyridinium borane complex (PBC) was added at the same moment as depurination was initiated, so as to achieve an immediate reduction of crosslinks. The process of reduction was subsequently completed by addition of sodium borohydride. This protocol fixes crosslinks to both lysine and histidine residues but since lysines are more numerous, it emphasises this crosslink and is termed for convenience only the 'LYS' protocol. In the second reduction protocol, no PBC was present during depurination: reduction of the crosslinks was achieved by the addition of borohydride only after completion of depurination. This procedure is selective for a small and rather particular subset.
of histone-DNA interactions (see Discussion). DNA crosslinks with histidine imidazoles predominate, though these are not the exclusive adducts formed. This is termed the 'HIS' protocol.

Soluble chromatin was prepared from crosslinked nuclei by mild micrococcal nuclease digestion; histones H1 and H5 were then removed by ion-exchange [14] and a second micrococcal nuclease digestion then used to produce nucleosomal core particles. The rationale for employing two sequential nuclease digestion steps was to avoid under-representation of the hyperacetylated fraction, known to display an enhanced sensitivity towards nucleases [1]. Chromatin solubilisation and H5/H1 depletion both act to equalise active and inactive structures by destroying vestiges of whatever special higher order structures pre-existing in the nuclei. It should be noted that histone-DNA contacts had already been fixed by crosslinking before these procedures. The hyperacetylated fraction was selected by mixing the derived core particles with anti acetyl-lysine antibodies [5,6] and immobilising the adducts on formalin-fixed S. aureus cells.

The immobilised hyperacetylated histones with covalently linked DNA were released from the S. aureus cells by SDS and subjected to diphenylamine/formic acid hydrolysis followed by digestion with micrococcal nuclease. The residual histones were labelled at the 5'-OH of the nucleotide fragment remaining at the site of crosslinking using T4 polynucleotide kinase and [γ-32P]-ATP. To separate the individual core histones from each other they were cut from an SDS gel after visualization by autoradiography. This permits a compete separation of histone peptidic regions crosslinked to nuclear DNA in: i—input, i.e. unfractionated core particles derived from crosslinked nuclei; b—bound core particles retained by the antibody; u—unbound core particles not held by the antibody. n—lanes representing the crosslinking patterns of core particles isolated from un-crosslinked nuclei and then subjected to the crosslinking procedure. The peptide numbering system conforms to those previously used in all cases for which the correspondence between bands is clear cut [11,16]. Some peptide bands, to avoid any bias, are assigned unique notations not necessarily implying their difference from the ones identified in a separate study (see text; Guschin et al., manuscript in preparation). Whenever the equivalence of two seemingly identical peptides was not immediately evident, they were assigned different notations, e.g., 3a2 and 3n2). Some notations are included for possible future reference. The integral intensity of peptide bands in any lane when compared with any other lane does not necessarily reflect the relative efficiency of crosslinking in each of the parallel experiments. Several bands easily observable on visual inspection of the autoradiograms have not reproduced well photographically.

Figure 1. Flow chart of the crosslinking and immunofractionation experiments leading to the 4 final samples b, i, l and n. See text for explanations.
Controls
The crosslinked peptides found in the antibody-bound fraction were compared with those from histones in the supernatant ('unbound'), and with those from the 'input' chromatin. In addition, the crosslinked peptides from these three fractions were compared with crosslinked peptides obtained from nucleosomes that had been isolated before being crosslinked.

It was important to be certain that the crosslinking and extraction procedures preceding antibody fractionation did not lead to a loss of acetyl groups. We therefore included 10 mM sodium butyrate at all stages of the preparation and checked with acetic acid/urea/Triton X-100 gels that the level of acetylation was not diminished by our procedures and that the antibody-bound fraction did indeed contain hyperacetylated histones (data not shown). Crosslinked nuclei displayed a normal micrococcal nuclease digestion pattern indistinguishable from that of nuclei that had not been crosslinked (not shown).

Comparative nucleotide-peptide mapping
Figure 2A shows the nucleotide-peptide patterns obtained for H3, H2(A plus B) and H4 histones using the 'HIS'-protocol and Figure 2B shows the equivalent patterns obtained with the 'LYS' protocol. For each histone, the peptide pattern from crosslinked nuclei that were used as 'input' (i) to the antibody fractionation is compared to that of the bound (b) and unbound (u) component; all these are compared to the crosslinking patterns of previously isolated nucleosomes (n).

The first observation of note is that for both protocols the nucleotide-peptide pattern of the unbound (u) fraction, which constitutes the bulk (95–97%) of the input chromatin, is very similar to that of the input chromatin (i). Although this equivalence is expected, the finding of good correspondence between these two patterns is a reassurance that the peptide patterns are largely free of artefacts resulting from the long and complex procedures used, particularly bearing in mind that the former was not subjected to antibody fractionation, unlike the latter. Also reassuring is the observation that the n fraction (likewise not subjected to immunofractionation) displays little deviation from the i and u peptide patterns. This correspondence is particularly strong for the HIS protocol, although some modifications can be seen in the n lanes with the LYS protocol, e.g. for histones H3 and H2, as also noted previously [15].

Histone H4
Using the HIS protocol the peptide pair T5, previously assigned to the dipeptide His(18)-Arg(19) crosslinked via histidine-18, predominates in the unbound, input and nucleosomal lanes, as expected from previous observations [11]. In the bound fraction however, the T12, T15 and T17 bands are of a similar intensity to the T5 pair and several bands of lower mobility that are very evident in the 'i' and 'n' lanes, e.g. T6f and T11 [11] and the strong band lying between them cannot be seen in the bound fraction. There is thus a considerable redistribution of intensity among the bands in the bound fraction and in particular the T5 pair is very much reduced relative to the other bands.

Histones H2(A + B)
Using the LYS protocol many crosslinked peptides can be seen, as expected for the two core histones most rich in lysines. Although some changes can be seen in the peptide pattern from the 'bound' fraction, as compared to u, i and n, the changes are not particularly striking. In contrast, use of the 'HIS' protocol shows very well defined differences in the bound fraction. For example, peptides 2n2, 2n3, 2n7, 2n15, 2n17 are present in the bound fraction as well as in i, but peptides such as 2n1, 2n10, and 2n11 are notable by their absence in b. The almost complete absence of band 2n10 is particularly noteworthy since this represents the C-terminal portion of H2A (see Discussion).

Histone H3
Significant differences between the bound and input fractions are observed using both protocols with the appearance of new bands and also the loss of others in the bound fraction. Using the 'HIS' protocol bands 3n1 and the 3n2 doublet are not observed in the 'bound' fraction although, for comparison, peptides 3n5, 3n7, and 3n13 are found in both the bound and unbound fractions. Conversely, bands 3a4 and 3a5, as well as 3a1 and 3a2, seem to be unique to the bound fraction. Peptides 3a4 and 3a5 could be the same as peptides 2a1 and 2n7, (seen as major components in the bound fraction of the H2 component), as a consequence of contamination of the eluted H3 band by components of the H2 fraction. Using the LYS protocol a number of bands are much reduced in intensity in the bound fraction, while at least one band is strikingly increased. Unfortunately, none of the H3 peptides have yet been assigned to regions of this histone.

DISCUSSION
Using the 'LYS' protocol nearly half of all the histone-DNA crosslinks obtained originate from the histone N-terminal tails [2,15], within which are located the acetyl groups. In hyperacetylated chromatin at least half, but certainly not all of the modifyable lysines are expected to be acetylated. The disappearance of some bands and emergence of new ones in the bound fractions, e.g. in the patterns of H3 and H4 using the 'LYS' protocol, can be interpreted as a major displacement and rearrangement of H3/H4-DNA contacts.

Changes in peptide pattern can however occur without major structural alterations since the presence of acetyl groups on a lysine sidechain makes it unable to crosslink to DNA and also renders it no longer a substrate for trypsin. Typical for the N-terminal tails, especially histones H3 and H4, is the situation in which there are two lysines in the sequence with no arginine between them. If one of the lysines can be crosslinked to DNA, while the other one can be acetylated, then in the event of acetylation a new and larger nucleotide-peptide would be generated by trypsin, with the loss of a smaller band, even though no new histone-DNA contact had been established. Thus the new bands in the bound fraction from the 'LYS' protocol may reflect the persistence of contacts via unmodified lysines close to acetylated lysines.

In the case of histone H4 the LYS protocol results in several changes in the banding pattern (e.g. the T5 pair) and no obvious reduction in the total number of bands. Since a good proportion of the bands must originate from the N-terminal tails, it follows that they are not completely removed from the DNA in the acetylated regions of the chromatin. For histone H3 however, the apparent reduction in the number of bands in the bound fraction using the LYS protocol (even allowing for a reduced loading) is indicative of fewer DNA contacts in the modified chromatin regions. In the case of the H2 histones the relatively unchanged pattern in the bound fraction using the LYS protocol...
indicates that there is no major remodelling of contacts in the N-terminal tails of these histones.

In contrast to the ‘LYS’ protocol, it has already been shown [2,15] that with the ‘HIS’ protocol the N-terminal tails of core histones contribute little to the pattern or number of crosslinked peptides. Changes in the ‘HIS’ peptide patterns therefore bear witness to changes in the interior of the nucleosome. The ‘HIS’ protocol appears to select a small subset of contacts, particular in that they are found at sites of sharp localized DNA bending [11,16 and D.Yu.Guschin, K.K.E., and A.D.Mirzabekov, manuscript in preparation]. Many of these contacts seem to be altered or aborted on transcriptional activation; in contrast, the ‘LYS’ protocol detects plentiful crosslinks in both active and silent chromatin domains [2,15].

The peptide pair T5, from histone, H4 is seen in the bound fraction with both protocols, but very much more weakly when compared to T6f, T15 and T17. This implies a loosening of the interaction between this region of H4 and the DNA in hyperacetylated chromatin and accords with genetic evidence from yeast that replacement of amino acids in this region (residues 16—19) leads to derepression of the otherwise constitutively silent mating loci HMLα and HMRα [17—19].

Of particular interest is the absence of peptide 2n10 in the bound fraction of histones H2 using the ‘HIS’ protocol. Peptide 2n10, by virtue of its relative mobility and abundance, is equivalent to peptide 1 of Ref. 16 and D.Y.Guschin, K.K.E., and A.D.Mirzabekov (loc cit), which was shown to represent a crosslink via His123 of histone H2A, a residue located six amino acids from the C-terminus of H2A. The almost complete absence of this peptide in the bound fraction indicates a loosening of DNA contacts in the C-terminal tail of H2A in hyperacetylated chromatin. This region of H2A is subject to considerable sequence variation, particularly in replacement H2A histones, and includes the site of ubiquitination at Lys119 and sites of phosphorylation at serines 122, 124, 129 (reviewed in [20]). It is also in the immediate vicinity of histone H1 at the entry/exit point of the nucleosome [21].

To what extent are the structural changes observed in acetylated chromatin to be directly attributed to the modification? Crosslinking in nuclei, as performed here, is different from a fully defined in vitro system of core histones plus DNA, in that other specific features of the active chromatin present in nuclei do not allow one to infer a direct cause-and-effect relationship. The recent in vitro observation that hyperacetylation facilitates the recognition by TFIIA of the 5S RNA gene assembled into nucleiosomes [22] suggests that hyperacetylation can exert its effects directly. It is therefore possible that the changes in crosslinking patterns are a direct consequence of acetylation at active loci. Acetylation at Lys16 of histone H4 could make a local contribution to the relaxation of histone binding in its vicinity and lead directly to the reduction in the intensity of the T5 peptide pair. The C-terminal tail of H2A is not subject to acetylation and the loss of peptide 2n10 in the hyperacetylated chromatin cannot therefore be due to nearby acetyl goups blocking the action of trypsin. The loss of this contact at a residue remote from the site of acetylation in H2A (lysine 5) indicates a concerted conformational change in hyperacetylated/active nucleosomes.

These data on an altered pattern of histone interactions with DNA in hyperacetylated chromatin are in very good accord with the findings of previous studies [2,15] which also documented histone-DNA crosslinks. This agreement, despite the widely different approaches (hybridization with two-dimensional ‘protein images’ in one case and anti-acetyl lysine immunofractionation here), are a reassurance that certain basic features of transcriptionally active chromatin have been revealed.

Finally, it is worth stressing that the broad correspondence between the peptide patterns obtained for isolated nucleosomes (n) and the total chromatin obtained from crosslinked nuclei (i) indicates that there is no major change in the mode of core histone attachment to the DNA as the 30nm supercoil is unwrapped and nucleosomes are excised with micrococcal nuclease. This observation confirms previous results [15]. That being so, specific features observed in the peptide patterns of the antibody-bound fraction are not simply due to some aspect of crosslinking within an extended nucleofilament, as distinct from the compact 30nm fibre, but must result from more specific structures characteristic of highly acetylated and presumably transcriptionally active regions.

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