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Distinctive patterns of histone H4 acetylation are associated with defined sequence elements within both heterochromatic and euchromatic regions of the human genome

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ABSTRACT

The pattern of histone H4 acetylation in different genomic regions has been investigated by immunoprecipitating oligonucleosomes from a human lymphoblastoid cell line with antibodies to H4 acetylated at lysines 5, 8, 12 or 16. DNA from antibody-bound or unbound chromatin was assayed by slot blotting. Pol I and pol II transcribed genes located in euchromatin were shown to have levels of H4 acetylation at lysines 5, 8 and 12 equivalent to those in input chromatin, but to be slightly enriched in H4 acetylated at lysine 16. In no case did the acetylation level correlate with actual or potential transcriptional activity. All acetylated histone H4 isoforms were depleted in non-coding, simple repeat DNA in heterochromatin, though the extent of depletion varied with the type of heterochromatin and with the isoform. Two single copy genes that map within or adjacent to blocks of paracentric heterochromatin are depleted in H4 acetylated at lysines 5, 8 and 12, but not 16. Consensus sequences of repetitive elements of the *Alu* family (SINES, enriched in R bands) were associated with H4 that was more highly acetylated at all four lysines than input chromatin, while H4 associated with *Kpn*I elements (LINES, enriched in G bands) was significantly underacetylated.

INTRODUCTION

The nucleosome core particle consists of 146 bp of DNA wrapped around an octameric complex of core histones (two each of H2A, H2B, H3 and H4) and is the basic structural unit of chromatin in all eukaryotic cells (1). In addition to this highly conserved structural role, nucleosomes are now known to be actively involved in the regulation of several fundamental cellular processes, including transcription, DNA replication and cell cycle progression (2–5). Regulatory functions are mediated, at least in part, by fine adjustments to the nucleosome through enzyme catalysed, post-translational modifications of the core histones. These include acetylation, phosphorylation, ADP ribosylation, ubiquitination and glycosylation (6,7).

Histone acetylation is one of the most frequent modifications and certainly the most extensively studied. It is ubiquitous in plants and animals. Acetylation of the core histones occurs at the ε amino groups of conserved lysine residues in the N-terminal region of each protein and is catalysed by a family of enzymes, the histone acetyltransferases (HATs). The modification is chemically stable but metabolically reversible through the activities of a second enzyme family, the histone deacetylases (HDACs). Both enzymatic activities are often found to be present in complex, multi-subunit assemblies that differ in substrate specificity, susceptibility to inhibitors and intracellular location (8–10). Genes encoding subunits of both HATs and HDACs have been cloned recently and shown to have homology to (or complete identity with) known regulators of transcription (8,11,12).

We have proposed that, because histone acetylation is involved in such a variety of cellular processes, it is likely that different functions will require selective acetylation of specific histones and even of specific lysine residues on individual histones (13,14). Direct support for this idea has come from the demonstration that H4 specifically diacetylated at lysines 5 and 12 is involved in post-replication chromatin assembly (15) and by recent studies on purified HATs showing that, *in vitro* at least, they are highly selective in the histone lysines they acetylate. In order to provide a more general means for testing this proposition we have prepared antisera that can distinguish histone isoforms acetylated at specific lysine residues (16,17). These have been used both for immunofluorescence microscopy of polytene and metaphase chromosomes (18–22) and immunoprecipitation of chromatin fragments (23). The former permits visualization of histone acetylation across relatively large chromatin domains and has shown that defined regions, and even whole chromosomes, can show characteristic patterns of histone acetylation (20), sometimes involving acetylation of specific lysines (18). The

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latter can be used to analyse acetylation of histones associated with particular genes or subregions within genes and can be used to relate histone acetylation to genomic structure and function, including actual or potential transcriptional activity (23).

In the present report we describe the use of antibodies specific for H4 acetylated at lysines 5, 8, 12 or 16 to immunoprecipitate oligonucleosomes from a human diploid lymphoblastoid cell line and thereby map acetylated H4 isoforms to defined regions of the genome. The results show that different regions are packaged as chromatin with widely differing levels of H4 acetylation. A striking finding is that GC-rich retroposon-derived sequences (SINES), characteristic of chromosomal R bands, are enriched in acetylated chromatin fractions, while GC-poor sequences (LINES), characteristic of G bands, are depleted. Coding DNA, irrespective of its transcriptional status, shows only a modest increase in acetylation (and only at lysine 16) compared with bulk chromatin. The results are consistent with immunofluorescence studies with antisera to acetylated H4 in which R bands label more strongly than G bands, but show that this differential labelling is due to differences in SINE/LINE content rather than coding DNA *per se*.

MATERIALS AND METHODS

Polyclonal antisera to acetylated histone H4 isoforms

Polyclonal antisera to acetylated H4 were raised in rabbits by immunization with synthetic peptides which correspond to the sequence in the N-terminal domain of histone H4 containing acetyl-lysine residues at defined positions. The preparation and characterization of these antisera have been described in detail elsewhere (16–18). For immunoprecipitation of unfixed chromatin each antiserum was affinity purified, as described previously (16–18,23)

Cultured cells

Normal B lymphocytes can be transformed and immortalized by infection with Epstein–Barr virus (24,25).The female lymphoblastoid cell line used in this study was provided by Prof. A.M.R.Taylor (University of Birmingham, UK) and had no detectable chromosomal aberrations. Cells were grown in RPMI medium (Gibco BRL) supplemented with 8% foetal calf serum (Gibco BRL) in an atmosphere of 5% CO_2 in air.

Preparation of chromatin from lymphoblastoid cells

Chromatin was isolated from lymphoblastoid cells as described previously (23). Cells were grown to a density of ~10^6 cells/ml and labelled for 16 h with 0.5 µCi/ml [^{3}H]thymidine (Amersham). Each immunoprecipitation experiment required 10^8 cells for sufficient yields of material. All steps were performed at 4°C in the presence of 5 mM sodium butyrate. Cells were harvested by centrifugation, washed, lysed with 0.5% Tween 40 and homogenized in a Dounce all-glass homogenizer with the A pestle. Homogenates were applied to a discontinuous 25–50% (w/v) sucrose gradient to isolate nuclei. The nuclear pellet was resuspended in digestion buffer (0.32 M sucrose, 50 mM Tris–HCl, pH 7.5, 4 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM PMSF, 5 mM sodium butyrate) to a final concentration of 0.5 mg DNA/ml (as determined by absorbance at $\lambda_{260\ nm}$).

Chromatin was released from nuclei by digestion with micrococcal nuclease (final concentration 75 U/ml; Pharmacia) at 37°C for 5 min. Digestion was stopped by addition of Na_2EDTA to a final concentration of 5 mM and cooling on ice. The preparation was centrifuged (11 600 *g*, 10 min) and the supernatant designated S1. The pellet was resuspended in lysis buffer (1 mM Tris–HCl, pH 7.4, 0.2 mM Na_2EDTA, 0.2 mM PMSF, 5 mM sodium butyrate), dialysed extensively and centrifuged to yield a solubilized chromatin fraction, designated S2. The extent of nuclease digestion was assessed by 1.2% agarose gel electrophoresis (26). Fractions S1 and S2 were pooled to form the input material for immunoprecipitations.

Immunoprecipitation of chromatin from cultured cells

The procedure for immunoprecipitation of unfixed chromatin has been described in detail elsewhere (23). All buffers contained 5 mM sodium butyrate. Briefly, affinity-purified antiserum (100–200 µl, containing 50–100 µg IgG) was added to 100–200 µg unfixed chromatin and the final volume made up to 1 ml with incubation buffer (50 mM NaCl, 20 mM Tris–HCl, pH 7.5, 20 mM sodium butyrate, 5 mM Na_2EDTA, 0.1 mM PMSF). The mixture was incubated for 16 h at 4°C, then treated with 200 µl 50% (v/w) slurry of protein A–Sepharose (Pharmacia) for 3 h at room temperature. After centrifugation the supernatant, containing antibody-unbound material, was retained and the protein A–Sepharose pellet was washed three times with wash buffer (50 mM Tris–HCl, pH 7.5, 10 mM EDTA, 5 mM sodium butyrate) containing increasing concentrations (50, 100 and 150 mM) of NaCl. Antibody-bound material was eluted from the protein A–Sepharose by treatment with 250 µl 1% SDS in incubation buffer for 15 min at room temperature. SDS in the bound fraction was then diluted to 0.5% with incubation buffer, to a final volume of 500 µl.

DNA was obtained from the input, unbound and bound fractions by two phenol/chloroform extractions and one chloroform extraction. DNA was ethanol precipitated using 5 µg glycogen as carrier and dissolved in 250 µl TE buffer (10 mM Tris–HCl, 2 mM Na_2EDTA, pH 7.4). All DNA samples were analysed by 1.2% agarose gel electrophoresis and incorporation of [^{3}H]thymidine was determined by scintillation counting.

Proteins from input, unbound and bound fractions were obtained from material in the organic phase of the first phenol/chloroform extraction of each sample (27). Carrier protein (5 µg bovine serum albumin), 0.01 vol. 10 M H_2SO_4 and 12 vol. acetone were added to precipitate protein at –70°C. After centrifugation protein pellets were washed in acidified acetone (1:6 100 mM H_2SO_4:acetone) and three times in dry acetone. Equal amounts of histone H4 were loaded on 15% SDS-containing polyacrylamide gels. Resolved proteins were transferred to Hybond-C nitrocellulose (Amersham) and histone H4 was detected by enhanced chemiluminescence (Amersham), as described previously (18,23).

Slot-blot analysis and Southern hybridizations

DNA samples were diluted in 0.6 M NaCl to equalize [^{3}H]thymidine counts, heat denatured at 95°C for 10 min and cooled on ice for 5 min. Small aliquots were taken at this point to retest [^{3}H]thymidine counts. A series of five serial doubling dilutions in ice-cold 2 M ammonium acetate was then carried out for each sample. Aliquots (200 µl) of each sample dilution were loaded in duplicate onto Hybond N^+ nylon membranes (Amersham) using a slot-blot manifold (BioRad). All slots were washed with 1 M

ammonium acetate before fixing the DNA on the filter with 0.4 M NaOH.

Hybridizations, with either end-labelled oligonucleotides or random primed DNA fragments, were performed as described previously (23) using standard procedures (26). The intensities of labelled slots were quantified using a PhosphorImager (Molecular Dynamics). Labelled membranes were stripped with boiling 0.5% SDS, followed by gradual cooling to room temperature. Membranes could be labelled and stripped ~10 times without significant loss of signal.

DNA probes

HGH 1 (human growth hormone 1), clone pHGH107, was obtained from the American Type Culture Collection (ATCC, reference no. 31538; US patent no. 4,342,832) and digested with *Hin*dIII and *Eco*RI to release a 591 bp cDNA fragment. ATCC literature cites the HGH 1 gene as a single copy gene at the cytogenetic location 17q22–q24. Telomeric DNA probe C36 [5′-$(CCCTAA)_6$-3′] was a gift from Dr Titia de Lange (Rockefeller University, New York, NY). The remaining oligonucleotide probes were as follows: heterochromatin probes het266 [5′-$(CCATT)_6$-3′], het405 (5′-GAA GAA GCT TTC TGA GAA ACT GCT TAG TG-3′) and het527 (5′-TCC AAA GCC CAT GTA GGC CGA GCC AAG ACA AGA GT-3′); SINE (*Alu* family consensus sequence) probe Alu450 (5′-AAA GTG CTG GGA TTA CAG G-3′); LINE (*Kpn*I family consensus sequence) probe Line B201 (5′-CAT GGC ACA TGT ATA CAT ATG TAA CWA ACC-3′). CpG island probe M2 was made from total human DNA selected on an MeCP2 column (28) and was a gift from Dr Sally Cross (Edinburgh University, UK).

Quinoline oxidoreductase (GenBank accession no. L13278) and p130/Rb-like protein 2 (GenBank accession no. X74594) are genes that we have mapped to pericentric heterochromatin of human chromosomes 1 and 16 respectively (cytogenetic locations 1q12 and 16q12). The GDB™ Human Genome Database version 6.0 (29) was used to query the location of cytogenetic regions 1q12 and 16q12 on an integrated linkage map (accessed on the World Wide Web at URL: http://gdbwww.gdb.org/jmqp/queryBy Posn.html). The range of linkage markers for each cytogenetic region was used to identify candidate, single copy genes with the integrated gene map (30) at http://www.ncbi.nlm.nih.gov/SCIENCE96/. IMAGE consortium cDNA clones of EST sequences (31) of the quinoline oxidoreductase and p130 genes, identified by IMAGE Consortium (Lawrence Livermore National Laboratory, CA) clone ID nos 489645 and 612408 and GenBank accession nos AA099529 and AA179202, were obtained from the UK Human Genome Mapping Project (HGMP) Resource Centre, Hinxton, Cambridge. The cDNA inserts from these clones were excised with *Eco*RI/*Not*I or *Eco*RI/*Xho*I to give fragments of size 456 and 462 bp, for quinoline oxidoreductase and p130 respectively. These probes both gave clean single bands by Southern analysis of restricted genomic DNA.

An EST was also identified for the human 28S rRNA gene (GenBank accession no. M11167) with IMAGE Consortium (LLNL) clone ID no. 342765. The insert of 414 bp was excised with *Eco*RI/*Not*I. All other DNA probes were as described previously (23).

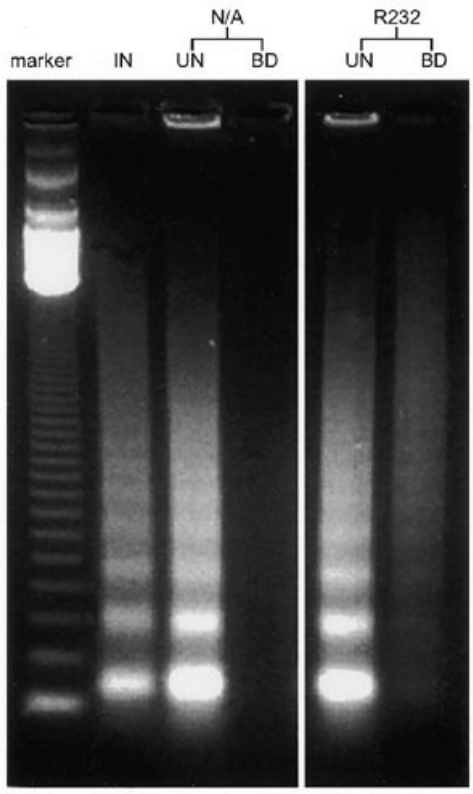

Figure 1. Size distribution of DNA isolated from input, unbound and bound chromatin fractions. Oligonucleosome fragments from input chromatin (IN) were immunoprecipitated with antibody R232 (specific to H4Ac8) or incubated without antibody (N/A). DNA was isolated from input, antibody-unbound (UN) or -bound (BD) fractions and resolved on a 1.2% agarose gel stained with ethidium bromide. The marker is a 123 bp ladder (Gibco BRL).

RESULTS

Immunoprecipitation results in selective enrichment of acetylated chromatin

Chromatin was prepared by limited micrococcal nuclease digestion of isolated nuclei. A pool of the first supernatant (S1) and the solubilized chromatin fraction (S2; see Materials and Methods) formed the input material for each immunoprecipitation experiment. The conditions of nuclease digestion were adjusted to give a high yield of soluble chromatin (78% of total DNA on average) while at the same time minimizing the possibility of selectively destroying the most nuclease-sensitive chromatin domains. The chromatin recovered by this procedure contained a high proportion of oligonucleosomes (Fig. 1).

Soluble chromatin was immunoprecipitated with affinity-purified antibodies specific for H4 acetylated at particular lysine residues (i.e. R13/16, R20/12, R232/8 and R41/5; the second number denotes the acetylated lysine residue recognized). H4 lysines are acetylated in a specific order in mammalian cells (16). In bulk chromatin lysine 16 is acetylated in *all* acetylated H4 isoforms, of which the mono-acetylated isoform ($H4Ac_1$) is by far the most frequent (32). Lysines 8 and 12 are acetylated only in the di-, tri- and tetra-acetylated isoforms ($H4Ac_{2–4}$), while lysine 5 is

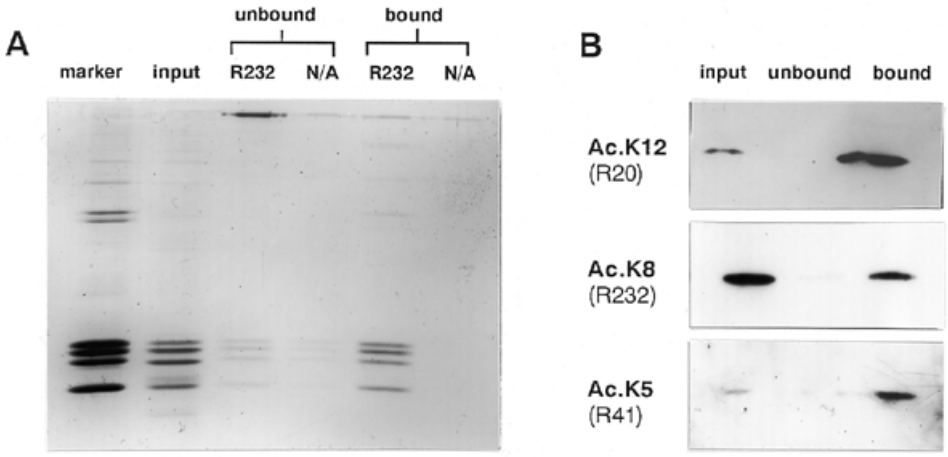

Figure 2. Analysis of proteins afer immunoprecipitation. (**A**) Proteins from input, unbound and bound fractions were isolated after immunoprecipitation in the absence of antibody (N/A) or in the presence of antibody R232. Core histones were resolved by 15% SDS–PAGE and stained with Coomassie blue. The amount of H4 in each fraction was quantified by scanning densitometry of each track. The marker is total acid-extracted histones from a human cell line. (**B**) Equal amounts of H4 from the input, unbound and bound fractions from immunoprecipitation experiments [calculated by densitometry, see (A)] were resolved by 15% SDS–PAGE. Western blotting and immunostaining with the appropriate antibody showed enrichment of H4 in the bound fraction. Immunocomplexes were detected by enhanced chemiluminesence (Amersham).

acetylated only in the tri- and tetra-acetylated forms. Because of this, antibodies to H4Ac16 are expected to immunoprecipitate chromatin fragments containing any acetylated H4 isoform, while R41/5 will precipitate only those containing the most highly acetylated forms. There will be exceptions to this general rule. For example, chromatin that is newly assembled after DNA replication is enriched in H4 acetylated specifically at lysines 5 and 12 (33). However, as this deposition-related pattern of acetylation persists for only a few minutes after DNA replication (33 and references therein), it is unlikely to significantly influence the pattern of acetylation across any particular region.

The efficiency of immunoprecipitation of the oligonucleosomes in input material was determined by quantifying the amount of [^{3}H]thymidine-labelled DNA in the unbound and bound fractions (Table 1). The total amount of material recovered after immunoprecipitation was, on average, 53% of the input material, while recovery of chromatin in the bound fraction varied with the antibody used. The percentage of recovered DNA in the bound fraction correlated with the expected frequency of histone H4 acetylation in bulk chromatin (i.e. H4Ac16 > H4Ac8,12 > H4Ac5). Thus antiserum R13/16, which recognizes all acetylated isoforms, precipitated more material than R41/5, which recognizes only the relatively rare tri- and tetra-acetylated isoforms.

DNA and proteins were isolated from the antibody-bound, -unbound and input fractions and analysed, respectively, by agarose gel electrophoresis, SDS–PAGE and Western blotting. For each experiment almost all the acetylated H4 recognized by the precipitating antibody was found in the bound fraction and very little in the unbound (Fig. 1B, lane 2). As noted previously by ourselves (23) and others (34), the DNA in a typical antibody-bound fraction was selectively enriched in longer oligonucleosomes (Fig. 1, compare lanes 3 and 4) and in histone H1 (Fig. 2A, lane 5). The reasons for this are unclear but are unlikely to be attributable to cross-reaction of the anti-AcH4 antibodies with H1 or to non-specific antibody binding. No evidence for such cross-reaction is seen on Western blots (data not shown) nor is there any consistent difference in the size of the chromatin fragments precipitated by the different antibodies. If cross-reaction were a significant problem then differences between antisera would be expected. Minimal precipitation occurred in the absence of antibodies or with preimmune antibodies, though we cannot exclude the possibility that larger fragments are pulled down through entrapment within immune complexes formed by genuine (i.e. specific) antibody–antigen reactions. However, the close correlation between the amount of chromatin precipitated by the different antibodies and that expected on the basis of the known frequency of acetylation at different H4 lysines (Table 1) strongly indicates that non-specific precipitation, however caused, is not a significant problem.

Table 1. Precipitation of chromatin with antibodies to acetylated H4 isoforms

Antiserum	Specificity	Chromatin precipitated (% input DNA)
R13/16	H4Ac16	14.7 ± 4.2 (n = 2)
R20/12	H4Ac12	11.2 ± 1.7 (n = 2)
R232/8	H4Ac8	8.8
R41/5	H4Ac5	1.6
No antibody		0.4 ± 0.07 (n = 4)

Chromatin from cultured human lymphoblastoid cells was immunoprecipitated with affinity-purified antibodies to acetylated H4 as described in the text. Input chromatin was radiolabelled by growing cells for 16–18 h prior to harvesting in medium containing [^{3}H]thymidine. DNA recovery was monitored by scintillation counting. Overall recovery after immunoprecipitation and DNA isolation from antibody-bound and -unbound fractions was 50–60%.

Equal amounts of DNA (based on [^{3}H]thymidine counts) from the input, unbound and bound fractions were applied to nylon membranes by slot blotting. All samples were applied as at least four doubling dilutions in duplicate. The filters were hybridized to ^{32}P-labelled oligonucleotides or DNA fragments corresponding to various coding and non-coding sequences and the level of hybridization quantified by phosphorimaging. The same filters were stripped and reprobed several times with different probes,

so the results for each probe are directly comparable. The labelling intensity of the bound DNA divided by that of the unbound DNA (i.e. the bound/unbound ratio) was used as a measure of the level of acetylation of H4 associated with specific DNA sequences. A bound/unbound (B/U) ratio of 1 indicates that the sequence is distributed equally between acetylated and non-acetylated chromatin. This level of acetylation is equivalent to that of the input (unfractionated) chromatin. Values >1 indicate relatively increased acetylation and values <1 represent depletion.

H4 acetylation associated with coding DNA in euchromatin

H4 acetylation along five representative pol II transcribed genes was tested. As shown in Table 2, acetylation of H4Ac16 was slightly increased in all cases (average B/U 1.47) whereas acetylation of lysines 5, 8 and 12 was the same as that of input chromatin (average B/U 0.99). Significantly, there was no correlation between the level of H4 acetylation and transcriptional activity of the genes tested. Bound/unbound ratios were the same in constitutively active genes (α-*tubulin* and c-*myc*) and silent genes (β-*globin*, *proinsulin* and *hgh*). In addition, there were no significant differences between acetylation along these coding regions and that in CpG islands (Table 3), which supports our previous study of H4 acetylation in the CpG island, promoter and coding regions of the c-*myc* gene (23). A very similar pattern of H4 acetylation (at least at lysines 8 and 16) was also seen in association with the transcriptionally hyperactive pol I transcribed 28S rRNA genes. The 28S rRNA genes are arranged in tandem repeats at the secondary constrictions of acrocentric chromosomes, which form the nucleolar organiser region (NOR) in interphase nuclei. These regions stain as strongly as other regions of euchromatin, but no more so, in metaphase chromosome spreads (A.M.Keohane and B.M.Turner, unpublished observations).

H4 acetylation associated with simple repeat DNA and coding DNA within or adjacent to such repeats

Simple repeat sequences characteristic of different types of constitutive heterochromatin were generally associated with low levels of H4 acetylation. However, the level varied from one repeat to another and, to a lesser extent, from one H4 lysine to another. All three sequences located in constitutive heterochromatin were associated with H4 that was underacetylated at all four lysines, with the core satellite III sequence showing the lowest acetylation and a *Sau*3A sequence the greatest (Table 3). Acetylation of H4 associated with the simple telomeric repeat $(CCTTAA)_n$ at lysine 16 was equivalent to that in coding regions, but slightly reduced at lysines 5, 8 and 12 (Table 3). Thus a general underacetylation of H4 is not a property of all types of chromatin containing simple sequence repeat DNA.

Table 2. Levels of acetylated H4 associated with genes in euchromatin transcribed by pol II

Acetylated H4 isoform		Euchromatin (pol II) genes						
		c-*myc*	β-*globin*	*pro-insulin*	*hgh*	α-*tubulin*	Average	SD
H4Ac16	(i)	1.30	1.28	1.15	1.41	1.43	1.31	0.11
	(ii)	1.55	1.49	1.93	ND	1.65	1.66	0.21
H4Ac12	(i)	0.93	0.69	0.91	ND	ND	0.84	0.13
	(ii)	0.89	0.90	1.04	1.18	1.09	1.02	0.12
H4Ac8		0.95	1.12	1.04	1.12	0.91	1.03	0.10
H4Ac5		1.17	1.14	0.88	ND	0.93	1.03	0.15

Amounts of each specific DNA sequence in the antibody-bound (i.e. acetylated) and -unbound fractions after immunoprecipitation with antibodies to acetylated H4 were determined by slot-blotting. Values in the table represent the ratio between the amount of each sequence in the antibody-bound and -unbound fractions. Values >1 indicate enrichment in acetylated H4 relative to bulk chromatin while values <1 indicate depletion. Rows labelled (i) and (ii) are results from separate experiments.

Table 3. Relative levels of H4 acetylation associated with different regions of the human genome

Acetylated H4 isoform		Pol II genes (average)	CpG islands	Pol I gene	Simple repeat sequences				Heterochromatin genes		SINEs	LINEs
				28S rRNA	sat III (het266)	alphoid (het405)	β/Sau3 (het527)	telomere (C-36)	QOR	p130/Rb-like	*Alu* family	*Kpn*I family
H4Ac16	(i)	1.31	1.30	0.97	0.15	0.38	ND	1.24	1.39	1.07	1.36	0.70
	(ii)	1.66	1.98	1.58	0.27	0.56	0.86	1.62	1.81	ND	1.53	1.08
H4Ac12	(i)	0.84	1.53	ND	0.34	0.31	ND	0.62	0.56	ND	0.92	ND
	(ii)	1.02	0.87	ND	0.43	0.68	0.82	ND	0.71	0.69	1.36	0.76
H4Ac8		1.03	1.11	0.96	0.38	0.28	0.26	0.89	0.95	0.81	1.49	0.67
H4Ac5		1.03	0.72	ND	0.17	0.57	0.53	0.81	0.47	ND	1.38	0.82

Figures in the Table are the antibody-bound/-unbound ratios after immunoprecipitation with antibodies to acetylated H4 (see text and Table 2 legend). Average values for five pol II genes are taken from Table 2. Values >1 indicate enrichment in acetylated H4 relative to bulk chromatin while values <1 indicate depletion. Rows labelled (i) and (ii) are results from separate experiments.

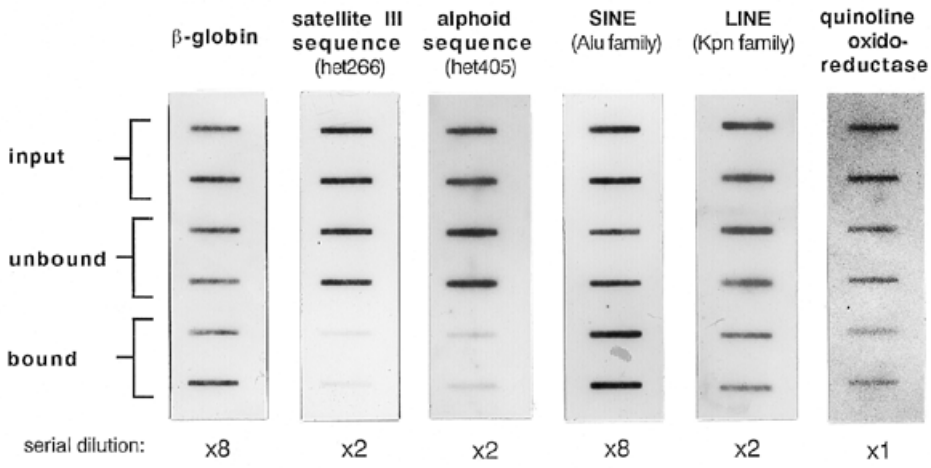

Figure 3. Slot-blot analysis of DNA after immunoprecipitation. DNA was isolated from input, unbound and bound fractions after immunoprecipitation with antibody R232. Equal amounts of DNA, based on the counts of incorporated [^{3}H]thymidine, were loaded in duplicate onto Hybond N$^+$ nylon membranes (Amersham). Five serial doubling dilutions were loaded, only one of which is shown (as indicated underneath each panel). The same membrane was used for labellings with the various indicated probes (see Materials and Methods).

The genes coding for quinoline oxidoreductase and p130/Rb-like protein map by linkage to regions within or adjacent to the blocks of heterochromatin at 1q12 and 16q12 (see Materials and Methods). These genes showed levels of acetylation at lysines 5, 8 and 12 that were (collectively) significantly below that of the five euchromatic pol II coding regions tested (Table 3, $P < 0.001$). Levels of acetylation at H4 lysine 16 were the same. This result suggests that these putative heterochromatin genes are depleted in the more highly acetylated H4 isoforms but retain a level of mono-acetylated H4 (the major isoform detected by R13/16) similar to that of euchromatic genes.

SINE-rich and LINE-rich chromatin differ in H4 acetylation

Moderately repetitive interspersed sequences are common in mammalian genomes. They are classified as either short interspersed repetitive sequences (SINES) or long interspersed repetitive sequences (LINES) (35,36). The major human LINES and SINES are the L1 (or *Kpn*I) family of sequences and the *Alu* family of sequences respectively (37). Both sequences are mobile genetic elements that have arisen by the process of retroposition and both can be transcribed into RNA. Oligonucleotides complementary to the consensus sequences of these repetitive elements were used as probes in immunoprecipitation experiments. The results (Fig. 3 and Table 3) show that SINES are relatively enriched in chromatin containing H4 acetylated at lysines 5, 8 and 12 compared with coding DNA (B/U ratios 1.29 ± 0.25, $n = 4$ and 0.99 ± 0.13, $n = 17$ respectively, $P < 0.01$), but not in chromatin acetylated at H4 lysine 16 (B/U ratios 1.45, $n = 2$ and 1.47 ± 0.23, $n = 9$ respectively). In contrast, LINE elements are relatively depleted in chromatin acetylated at any of the four acetylatable H4 lysines, including lysine 16, in comparison with coding DNA (B/U ratios 0.81 ± 0.16, $n = 5$, and 1.16 ± 0.28, $n = 26$ respectively, $P < 0.02$). The results indicate an increase in the most highly acetylated H4 isoforms (i.e. H4Ac$_{2–4}$) in SINE-rich regions of the genome and an overall reduction in H4 acetylation in LINE-rich regions.

DISCUSSION

Specificity of histone acetylation in human chromatin

Indirect immunofluorescence microscopy has been used to define the distribution of acetylated histones along mammalian metaphase chromosomes. The distribution is strikingly non-random. Centric heterochromatin and the blocks of heterochromatic DNA on the proximal long arms of chromosomes 1, 9 and 16 and on Yq are marked by very low levels of acetylation of all four core histones (19). In contrast, alternating brightly and weakly fluorescent bands are seen along the chromosome arms and correspond, in general terms, to R bands and G bands respectively (38). In female cells the inactive X chromosome (Xi) is also underacetylated throughout its length, with the exception of the pseudoautosomal region at Xp22 and two other narrow bands (20) which remain brightly fluorescent in metaphase chromosomes. In all studies so far antisera to the acetylated isoforms of all four core histones and to H4 acetylated at one or other of lysines 5, 8, 12 and 16 have all given similar, though not always identical, results (20, 21). As yet immunofluorescence analysis of mammalian chromosomes has not revealed any examples of histone-specific or lysine-specific acetylation comparable with the localization in *Drosophila* of H4Ac16 on the male X chromosome or H4Ac12 on centric β-heterochromatin (18).

However, while the immunofluorescence approach can define the broad distribution of histone acetylation across metaphase chromosomes and can provide a rapid and accurate assessment of H4 acetylation in both normal and aberrant chromosomes (39), it lacks the resolution necessary to determine levels of acetylation along specific genes or at levels below that of the chromosome band. It is also applicable only to mitotic (primarily metaphase) chromosomes, raising the possibility that the patterns seen may be peculiar to this rather unrepresentative phase of the cell cycle. The immunoprecipitation approach addresses both these limitations by providing resolution at the single gene level (or below) and, more importantly, by being applicable to cells at all stages of the cell cycle.

The results presented here provide two indications that lysine-specific H4 acetylation may be involved in the regulation

of mammalian chromatin. The first is the modest but consistent increase in H4Ac16 on all types of coding DNA, which presumably reflects an increase in the level of mono-acetylated H4 in these regions. It is interesting to note that in the yeast *Saccharomyces cerevisiae,* of the four acetylatable H4 lysines, lysine 16 is the only one whose acetylation can, on its own, prevent silencing of the mating type genes (40,41 and references therein). Both of these observations suggest that H4 lysine 16 has a pivotal role in determining the potential of coding DNA for expression or silencing. The second indication comes from the variation in patterns of H4 acetylation on different types of heterochromatin. For example, H4 associated with satellite III sequences (probe het266), present in the centric heterochromatin of several human chromosomes (42), is underacetylated at all four lysines. In contrast, the sequences recognized by probe het527, which are found in some major blocks of β-heterochromatin, particularly those adjacent to the simple sequence DNA satellite III (het266) contained in the centromeric chromatin of chromosome 9 (43), are strongly underacetylated at lysine 8, but show levels of acetylation only slightly below those of euchromatin at lysines 12 and 16. Such differences cannot easily be explained by shifts in the relative amounts of the mono-acetylated and more highly acetylated isoforms and suggest that regional differences in the levels of $H4Ac_1$ and $H4Ac_{2–4}$ are overlaid with more subtle lysine-specific differences. They are more consistent with the possibility that lysine-specific acetylation plays a role in packaging of different types of heterochromatic DNA, perhaps analogous to that played by H4 lysine 12 acetylation in *D.melanogaster* (18) and *S.cerevisiae* (44).

At first sight the variation in lysine-specific H4 acetylation associated with β-heterochromatin seems to be only partly consistent with the observation that in metaphase chromosome spreads the heterochromatin block on chromosome 9 labels weakly with antisera to *all* acetylated H4 isoforms (19; B.M.Turner, unpublished observations). A likely explanation is that levels of acetylation on certain types of heterochromatin are reduced as cells enter mitosis. This suggestion is consistent with the general deacetylation of core histones as cells enter mitosis (45) and with the observation that levels of acetylation along heterochromatin can vary as cells differentiate (23) and move through the cell cycle (L.P.O'Neill and B.M.Turner, unpublished observations).

Histone acetylation in coding and non-coding regions

We have shown previously by immunoprecipitation of chromatin from the aneuploid human cell line HL60 that there is no correlation between the overall level of histone acetylation and actual or potential transcriptional activity (23). This result is confirmed by the present experiments. All five pol II coding regions tested showed the same pattern of H4 acetylation irrespective of their transcriptional status, namely levels of acetylation at H4 lysines 5, 8 and 12 that were indistinguishable from those in bulk chromatin and a relative increase in acetylation at lysine 16. The latter is consistent with a selective increase in the mono-acetylated isoform, $H4Ac_1$. The same pattern of H4 acetylation was also seen in the single pol I gene tested, namely that encoding 28S rRNA. Thus the euchromatin genes tested showed a remarkably consistent pattern of H4 acetylation, despite having rather different chromatin environments: β-globin lacks a CpG island and maps within a G band, while the other four pol II genes are all R band genes that are associated with a CpG island (46,47). The results suggest that the pattern of histone acetylation along coding regions is, at least in euchromatin, independent of the surrounding chromatin. This was tested further by examining two genes that map within or adjacent to the large blocks of heterochromatin at 1q12 and 16q12. These genes were found to retain the relative enrichment in H4Ac16 characteristic of coding regions, but to show a significant drop in acetylation at lysines 5, 8 and 12 (presumably reflecting a relative depletion in hyper-acetylated H4). It is tempting to speculate that depletion of the more highly acetylated H4 isoforms in these genes is due to their proximity to underacetylated heterochromatin, while their relatively high level of acetylation at H4 lysine 16 is necessary to maintain their transcriptional competence. Testing of additional genes that map within or adjacent to heterochromatin will show whether or not this is a general rule.

R bands are characterized by being relatively rich in coding DNA, GC base pairs, SINE sequences and acetylated H4, while being relatively poor in LINE sequences. G bands, in contrast, are relatively poor in coding DNA, SINES and acetylated H4 but are rich in LINES and AT base pairs (46,47). A correlation has been noted between SINE density and gene density along the human genome, with T bands being particularly rich in both genes and SINES (48). This correlation may reflect the tendency of SINES to be located within introns (47). The results presented here suggest that the relatively high level of hyperacetylated H4 (i.e. the di-, tri, and tetra-acetylated isoforms) detected by immuno-fluorescence at R bands in general (20), and T bands in particular (38), is attributable more to their relatively high SINE density than to high levels of coding DNA itself.

It was the aim of the immunoprecipitation experiments described here to define the steady-state patterns of H4 acetylation across defined regions of the human genome. In order to do this we used a combination of nuclease digestion and solubilization conditions that minimized the possibility of selective DNA loss while still giving a high yield of soluble chromatin, usually ~80%. Earlier studies, with a rather different objective, namely to compare the structure and composition of transcriptionally active and inactive chromatin fractions, consistently used highly selected chromatin subfractions comprising only a small proportion of total chromatin (34,49). We suggest that the use of such very different starting chromatin preparations is a likely explanation for the fact that some previous experiments, unlike those reported here or earlier by us (23), have shown that chromatin fractions containing high levels of acetylated histones are also enriched in transcribed or transcribable genes. Such differences may indeed exist within highly selected chromatin subfractions, but may not be present when the experiment is carried out with essentially unselected, bulk chromatin. The lack of correlation between acetylation and transcriptional status noted here cannot easily be attributed to deficiencies in the immunoprecipitation procedure. The clear correlation between the amount of chromatin precipitated by each antibody and the relative frequencies of acetylation of H4 lysines 5, 8, 12 and 16 (Table 1) is a strong argument against the existence of excess non-specifically bound material in the antibody-bound fraction. In addition, non-specific chromatin precipitation cannot explain the finding that a large proportion of coding DNA, irrespective of transcriptional status, remains in the unbound fraction, despite almost complete precipitation of acetylated H4 (Fig. 2).

These considerations are also relevant to the initially puzzling finding that chromatin immunoprecipitated with antibodies to acetylated H4 is consistently enriched in larger oligonucleosomes. This apparently runs counter to the generally accepted idea that more highly acetylated chromatin fractions are more susceptible to nuclease digestion. In fact, the relationship between histone acetylation and the rate of digestion with micrococcal nuclease is complex. While it appears to be the case that, in some cells at least, a small chromatin fraction is both highly acetylated and relatively rapidly digested with micrococcal nuclease (see for example 50 and references therein), the same experiments show that a significant proportion of highly acetylated chromatin is *not* rapidly digested. In fact, in one fractionation scheme chromatin that remained in the pellet after digestion, presumably the most nuclease resistant, was just as enriched in highly acetylated H4 as the most rapidly digested S1 fraction (50). It should also be noted that in some experiments setting out to analyse the relationship between histone acetylation and nuclease sensitivity levels of histone acetylation were enhanced by exposure of growing cells to deacetylase inhibitors such as sodium butyrate (51,52). Treatments that result in artificial overall histone hyperacetylation may create relationships that do not exist in untreated cells (50). So, while we cannot yet completely exclude the possibility that enrichment of the antibody-bound fraction in larger oligonucleosomes is an artefact of the experimental protocol, it is also possible that it reflects the existence of an acetylated but micrococcal nuclease-resistant chromatin fraction. Experiments to test this are in progress.

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