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Gabriela Camporeale
University of Nebraska-Lincoln

Anna M. Oommen
University of Nebraska-Lincoln

Jacob B. Griffin
University of Nebraska-Lincoln

Gautam Sarath
University of Nebraska-Lincoln, Gautam.sarath@ars.usda.gov

Janos Zempleni
University of Nebraska-Lincoln, jzempleni2@unl.edu

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K12-biotinylated histone H4 marks heterochromatin in human lymphoblastoma cells^{☆,☆☆}

Gabriela Camporeale^{a,1}, Anna M. Oommen^{a,1}, Jacob B. Griffin^a,
Gautam Sarath^b, Janos Zemleni^{a,*}

^aDepartment of Nutrition and Health Sciences, University of Nebraska-Lincoln, Lincoln, NE 68583-0806, USA

^bUSDA-ARS and Department of Entomology, University of Nebraska-Lincoln, Lincoln, NE, USA

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Abstract

Covalent modifications of histones play crucial roles in chromatin structure and genomic stability. Recently, we reported a novel modification of histones: biotinylation of lysine residues. Here we provide evidence that K12-biotinylated histone H4 (K12Bio H4) maps specifically to both heterochromatin (alpha satellite repeats in pericentromeric regions) and transcriptionally repressed chromatin (γ-G globin and interleukin-2) in human lymphoblastoma cells. The abundance of K12Bio H4 in these regions was similar to that of K9-dimethylated histone H3, a known marker for heterochromatin. Likewise, K8-biotinylated histone H4 (K8Bio H4) mapped to heterochromatin, but the relative enrichment was smaller compared with K12Bio H4. Stimulation of interleukin-2 transcriptional activity with phorbol-12-myristate-13-acetate and phytohemagglutinin caused a rapid depletion of K12Bio H4 in the gene promoter. These data are consistent with a novel role for biotin in chromatin structure and transcriptional activity of genes.

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Keywords: Biotin; Chromatin immunoprecipitation; Heterochromatin; Histone; Human

1. Introduction

DNA-binding proteins such as histones H1, H2A, H2B, H3 and H4 play crucial roles in the regulation of chromatin structure and maintenance of genomic stability. Nucleosomal core particles in eukaryotes are composed of 147 base pairs (bp) of DNA wrapped around an octamer of core histones, consisting of two H2A-H2B dimers and one H3-H3-H4-H4 tetramer; linker histone H1 associates with the DNA connecting two core particles [1]. N-terminal tails of

histones are exposed at the nucleosomal surface and are targets for a multitude of covalent modifications such as acetylation and methylation of lysine residues and phosphorylation of serine residues [2,3]. These modifications form the basis for epigenetic events such as those regulating the transcriptional activity of genes and DNA repair processes. For example, K9-dimethylated histone H3 (K9Me2 H3) is associated with heterochromatin such as in major and minor satellite repeats in pericentromeric regions, and transposons in humans and mice [4,5], whereas K4-trimethylated histone H3 (K4Me3 H3) is associated with promoter regions in euchromatic genes [6,7]. Methylation of CpG islands in gene promoters is another epigenetic event mediating gene silencing and usually colocalizes with K9Me2 H3 in chromatin [4].

We recently identified a novel modification of histones by using mass spectrometry, synthetic peptides and biotinylation site-specific antibodies: binding of the vitamin biotin to ε-amino groups in lysine residues [8]. Specifically, the following biotinylation sites have been identified: K9, K13, K125, K127 and K129 in histone H2A [9], K4, K9 and K18 in histone H3 [10] and K8 and K12 in histone H4

Abbreviations: bp, base pairs; ChIP, chromatin immunoprecipitation; ELISA, enzyme-linked immunosorbent assay; HCS, holocarboxylase synthetase; PCR, polymerase chain reaction.

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^{☆☆} J.Z. has licensed anti-K12Bio H4 to Upstate. The other authors declare no conflict of interest.

* Corresponding author. Tel.: +1 402 472 3270; fax: +1 402 472 1587.

E-mail address: jzemleni2@unl.edu (J. Zemleni).

¹ G. Camporeale and A. M. Oommen contributed equally to this work.

Table 1
Specificity of monoclonal antibody to K8Bio H4

Probe	Target peptide	
	K8Bio [CKGGK(Bio)GLGKGGA]	K12Bio [CKGGKGLGK(bio)GGA]
Optical density (450 nm)		
Anti-K8Bio	3.4±0.6	0.8±0.1
PBS/Tween	0.1±0.01	0.1±0.02
Preimmune serum	0.1±0.03	0.1±0.03

Microtiter wells were coated with K8- and K12-biotinylated peptides based on human histone H4. Peptides were probed using monoclonal antibody to K8Bio H4 by ELISA. Negative controls were generated by probing peptides with phosphate-buffered saline (PBS) containing 0.05% Tween and preimmune serum.

[8]. The existence of biotinylated histones in human cells has been confirmed by mass spectrometry [11]. Biotinylation of histones is catalyzed by biotinidase [12] and holocarboxylase synthetase (HCS) [13] in two apparently independent reactions. The biological functions of most biotinylation sites are unknown. Preliminary evidence has been provided that biotinylation of K8 and K12 in histone H4 might play roles in the cellular response to double-strand breaks in DNA and chromosome condensation during the M phase of the human cell cycle [14–16]. More importantly, biotinylated core histones appear to be associated with transcriptionally silent chromatin in chicken erythrocytes [17]. Here, we provide evidence that K12-biotinylated histone H4 (K12Bio H4) and, perhaps, K8-biotinylated histone H4 (K8Bio H4) are markers for heterochromatin in pericentromeric regions and transcriptionally repressed chromatin in human lymphoblastoma (Jurkat) cells.

2. Materials and methods

2.1. Cell culture

Jurkat cells (clone E6-1) and JAr choriocarcinoma cells were obtained from ATCC (Manassas, VA). Cells were cultured in humidified atmosphere (37°C, 5% CO₂) as described [18]. Jurkat cells express interleukin-2 rapidly in response to appropriate stimulation [18,19]. Here, in some experiments cells were stimulated with 50 µg/L of phorbol-12-myristate-13-acetate (PMA) and 2 mg/L of phytohemagglutinin (PHA) for 2 h to change the status of the *interleukin-2* gene from transcriptionally repressed chromatin to transcriptionally active chromatin.

Biotinylation of histones is mediated by HCS [13]. Here, we generated HCS-deficient Jurkat cells by using small interfering RNA (siRNA) and the pSilencer 4.1-CMV neo vector (Ambion, Austin, TX); negative controls included siRNA targeting glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and a hairpin with limited homology to sequences in the human genome. Jurkat cells were transfected with the various pSilencer 4.1-CMV neo vectors, and stably transformed cells were selected using 0.3 g/L G418 for 10 days. Knockdown of HCS messenger RNA (mRNA)

was confirmed by using real-time polymerase chain reaction (PCR) as described below; abundance of HCS protein was quantified by Western blot analysis using an antibody described previously [9]. Here, HCS-deficient cells were used as a control in chromatin immunoprecipitation (ChIP) assays; a detailed account of genotypes and phenotypes associated with holocarboxylase deficiency will be presented in a separate article.

2.2. Chromatin immunoprecipitation assay

ChIP assays were conducted as described [20] with minor modifications. Briefly, Jurkat cells were cross-linked with 0.27 mol/L formaldehyde at room temperature for 15 min when cross-linking was quenched by using 0.125 mol/L glycine. Cells were collected by centrifugation and resuspended in lysis buffer (5 mmol/L PIPES, pH 8.0, 85 mmol/L KCl, 0.5% NP40) with protease inhibitors; cells were incubated at 4°C for 10 min with vortexing. Nuclei were collected by centrifugation and resuspended in nuclei buffer (50 mmol/L Tris, pH 8.1, 10 mmol/L EDTA, 35 mmol/L SDS). Samples were chilled on ice and DNA was sheared by using a Branson Sonifier (three 30-s pulses on ice at 60% amplitude with 60 s in between pulses) to produce fragments of approximately 1000 bp. Samples were centrifuged and the chromatin solution was precleared by using Immobilized Protein A on Trisacryl GF-2000 (Pierce; Rockford, IL) at 4°C for 2 hours. Aliquots were used for generating input DNA (without antibody precipitation) and for immunoprecipitation with antibodies at 4°C overnight. The following antibodies were used for ChIP assays. The

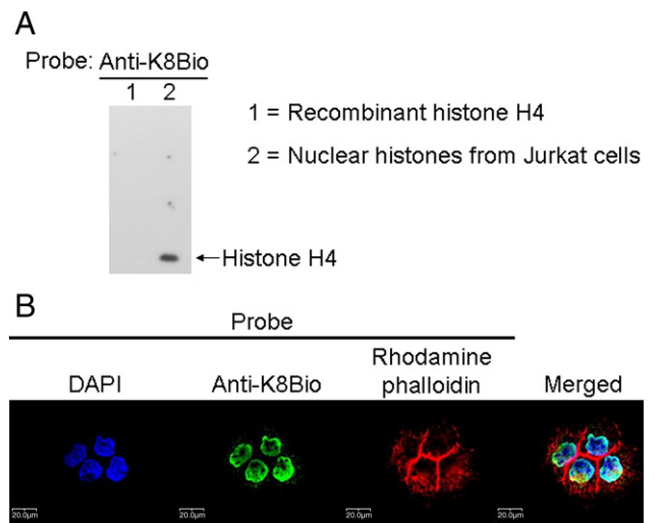


Fig. 1. Specificity and cellular localization of monoclonal antibody to K8Bio H4. (A) Monoclonal antibody to K8Bio H4 binds but does not cross-react with recombinant, nonbiotinylated histone H4 (Lane 1) but binds to biotinylated histone H4 in an extract of bulk histones from Jurkat cells (Lane 2). (B) Monoclonal antibody to K8Bio H4 localizes to the nucleus in JAr choriocarcinoma cells. The nuclear compartment was stained with DAPI, histones were stained using anti-K8Bio H4, and the cytoplasm was stained by using rhodamine phalloidin. The image entitled “Merged” was created by overlaying images obtained by staining with DAPI, antibody and rhodamine phalloidin.

Table 2

PCR primers used for quantification of DNA from ChIP experiments (denoted “ChIP”) and transcripts (denoted “Trans”)

Target	Experiment	Size (bp)	Forward primers		Reverse primers	
			Location ^a	Sequence	Location	Sequence
ADH5	ChIP and Trans	146	+606	5'-GCATAATTGAGCCTACGCC-3'	+732	5'-GCAGAGGTGTTTGTACGTG-3'
Chr1alpha	ChIP ^b and Trans	136	N/A	5'-TCATTCCCACAACTGCGTTG-3'	N/A	5'-TCCAACGAAGGCCACAAGA-3'
Chr4alpha	ChIP and Trans	139	N/A	5'-CTGCACTACCTGAAGAGGAC-3'	N/A	5'-GATGGTTCAACACTCTTACA-3'
GAPDH	ChIP	293	+1399	5'-CCCAACTTTCCCGCTCTC-3'	+1692	5'-CAGCCGCTGGTTCAACTG-3'
GAPDH	Trans	323	+28	5'-ACCACAGTCCATGCCATCAC-3'	+350	5'-GAGACCACCTGGTGCTCAG-3'
γ-G glob	ChIP and Trans	195	+84	5'-TCTACCCATGGACCCAGAGGT-3'	+278	5'-CCACATGCAGCTTGTCTCAGT-3'
HCS	Trans	101	+1	5'-ATGGAAGATAGACTCCACAT-3'	+102	5'-TGAGACCTGATCCTTAACCTCC-3'
IL-2prom	ChIP	350	-459	5'-CCACCCCTTAAAGAAAGGA-3'	-109	5'-ATTGTGGCAGGAGTTGAGGT-3'
IL-2, 5' end	ChIP	257	+29	5'-TGCAACTCCTGTCTTGCATT-3'	+286	5'-GAATCCCAAACTCACCAGGA-3'
IL-2, 3' end	ChIP	232	+6340	5'-AACCCAGATTACCTCAAGGTGGCA-3'	+6570	5'-AGTCCCAGCAGGAAATAGATGGCA-3'
IL-2prom2	ChIP ^c	286	-387	5'-GAGCTATCACCTAAGTGTGGGCTAA-3'	-101	5'-TGTGGCAGGAGTTGAGGTTACTGT-3'
IL-2	Trans	390	+29	5'-TGCAACTCCTGTCTTGCATT-3'	+419	5'-ATGGTTGCTGTCTCATCAGC-3'

ADH5=aldehyde dehydrogenase 5 (GenBank AY987960); Chr1alpha=chromosome 1 alpha satellite repeats (GenBank M26919); Chr4alpha=chromosome 4 alpha satellite repeats (GenBank M38467); γ-G glob=gamma-G globin (GenBank X55656); GAPDH=glyceraldehyde-3-phosphate dehydrogenase (GenBank AY340484 for ChIP; GenBank BC013852 for RT-PCR); HCS=holochoylase synthetase (GenBank NM_000411); IL-2prom and IL-2prom2=interleukin-2 promoter (GenBank AJ006884); IL-2, 5' end=5' end of the interleukin-2 gene (GenBank X00695); IL-2, 3' end=3' end of the interleukin-2 gene (GenBank AF359939); IL-2, interleukin-2 (GenBank BC066257); N/A=not applicable.

^a Relative to transcription start site (=+1).

^b These primers were used for both semiquantitative and real-time PCR.

^c These primers were used only for real-time PCR.

polyclonal antiserum against K12Bio H4 has been characterized before [8]. This antiserum is highly specific for K12Bio H4 [8]; titration experiments suggested that this antiserum has an affinity for K12Bio H4 that is at least 45 times greater than the affinity for K8Bio H4. The antiserum against K8Bio H4 is also very specific for biotinylated histone H4 (as opposed to other classes of histones and nonbiotinylated histones), but shows some cross-reactivity with K12Bio H4 [8]; specifically, the antiserum against K8Bio H4 has an approximately three times greater affinity for a synthetic peptide based on K8Bio H4 than for a peptide based on K12Bio H4. In select experiments we used a monoclonal antibody to K8Bio H4 (see below) to confirm findings made by using the polyclonal antibody to K8Bio H4; these samples were incubated with a goat anti-mouse secondary antibody (Sigma, St. Louis, MO) for 1 h subsequent to incubation with the monoclonal antibody. Affinity-purified antibodies against K9Me2 H3 and K4Me3 H3 were purchased from Abcam (Cambridge, MA). Protein A-purified rabbit IgG against K12Ac H4 was purchased from Upstate (Lake Placid, NY). Affinity-purified rabbit IgG to the C-terminus in histone H4 was purchased from Abcam (cat. no. ab10158). Chromatin was precipitated for 2 h using Immobilized Protein A Trisacryl GF-2000. Cross-linking was reversed by using 200 mmol/L sodium chloride (65°C overnight), and RNA was removed by using RNase A (10 g/L). Finally, protein was digested with Proteinase K (55°C, 2 h) and DNA was purified by using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). Each immunoprecipitation was repeated three times.

2.3. Monoclonal antibodies

A monoclonal antibody to K8Bio H4 was generated in the monoclonal antibody facility at the University of

Nebraska Medical Center (Omaha, NE) by using routine procedures [21]. The antibody facility is approved by the Institutional Animal Care and Use Committee and the U.S. Department of Agriculture. No procedures causing pain to the animal were performed without the use of appropriate anesthesia and subsequent analgesia. For injection into mice we purchased a K8-biotinylated peptide based on amino acids 5 through 15 in histone H4 (University of Virginia Biomolecular Research Facility, Charlottesville, VA); the peptide contained an additional N-terminal cysteine for conjugation to keyhole limpet hemocyanin (Pierce) before injection: CKGGK(biotin)GLGKGA. In addition, a K12-biotinylated peptide [CKGGKGLGK(biotin)GGA] was obtained for specificity testing by enzyme-linked immunosorbent assay (ELISA) described below. Peptide identities and purities were confirmed by using mass spectrometry and high-performance liquid chromatography. K8-biotinylated peptide was injected into mice, and hybridoma cells secreting antibody specific for K8Bio H4 were identified by ELISA (see below). IgG rather than IgM accounted for anti-K8Bio H4, as judged by Western blot analysis using a secondary Fc-specific antibody against IgG (data not shown). IgG secreted by hybridoma cells was purified using affinity chromatography. Briefly, bovine IgG introduced by fetal bovine serum (culture media) was removed by using a goat anti-bovine IgG column. Mouse IgG in the flow-through was purified by using a protein G column; IgG was eluted with 100 mmol/L glycine, pH 2.7. IgG was stabilized by adjusting the concentrations of Tris and NaCl to 40 and 150 mmol/L, respectively (pH 7.0).

The specificity of monoclonal anti-K8Bio H4 for distinct biotinylation sites was tested by ELISA. Briefly, K8-biotinylated and K12-biotinylated peptides were conjugated to bovine serum albumin by using Imject Activated BSA

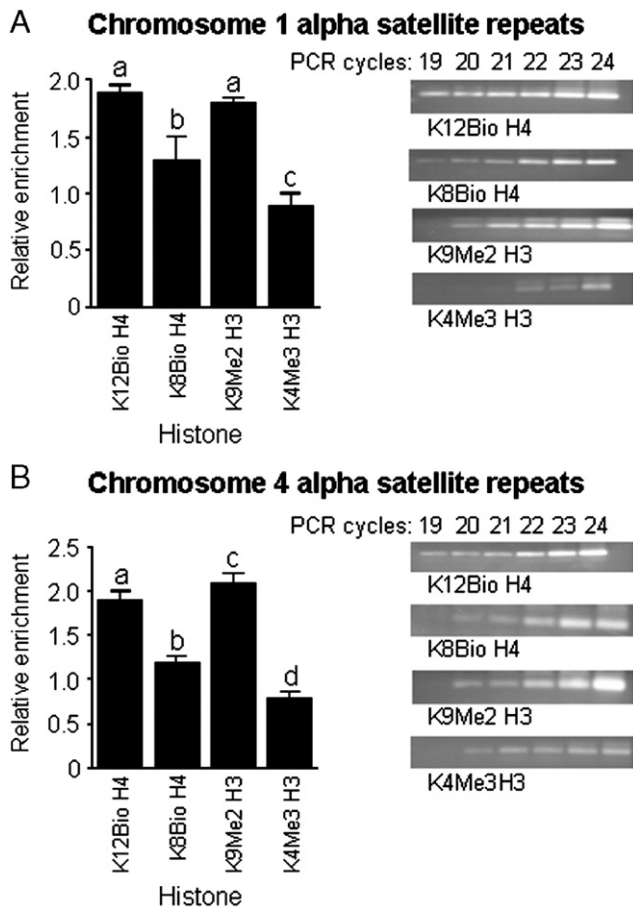


Fig. 2. Relative enrichment of heterochromatin by ChIP assay in Jurkat cells. Chromatin was immunoprecipitated using antibodies to K12Bio H4, K8Bio H4, K9Me2 H3 and K4Me3 H3. DNA was amplified by semiquantitative PCR, using primers specific for alpha satellite repeats on chromosomes 1 (A) and 4 (B); only values from within the exponential phase of amplification were considered for quantitative analysis. Bar graphs depict the relative enrichment of precipitated DNA compared with input DNA (mean \pm S.D., $n=3$). ^{a,b,c,d} Bars not sharing the same letter are significantly different ($P<0.05$). Gels depict representative examples from PCR.

(Pierce) according to the manufacturer's instructions. Microtiter plates were coated with albumin-conjugated peptides, and unspecific adsorption sites were blocked by using a buffer containing 0.1 mol/L HEPES (pH 7.0), 1 mol/L NaCl, 0.01% (wt/vol) bovine serum albumin and 0.05% (vol/vol) Tween 20 [22]. Antibody binding was quantified following routine ELISA procedures, using 0.66 μ g of anti-K8Bio H4 per well. Binding of monoclonal antibody was quantified by using goat anti-mouse IgG (Fc specific) peroxidase conjugate (Sigma; 28 μ g/L final concentration) and 3,3',5,5'-tetramethylbenzidine ("Sureblue microwell peroxidase substrate," KPL, Gaithersburg, MD). Blanks were prepared by using mouse preimmune serum and IgG-free buffers. ELISA data are consistent with the notion that the monoclonal antibody specifically targets K8Bio H4 (Table 1).

Antibody specificities were further tested by Western blot analysis as described [8]. Recombinant (nonbiotinylated)

histone H4 (Upstate) was probed with monoclonal anti-K8Bio H4 (8 μ g/L final concentration) to determine whether the antibody cross-reacts with nonbiotinylated histone H4. Histone extracts from Jurkat cells contain biotinylated histones H1, H2A, H2B, H3 and H4 in similar quantities [23]; extracts were probed with monoclonal anti-K8Bio H4 to identify potential cross-reactivity with histones H1, H2A, H2B and H3. In all Western blot experiments, goat anti-mouse IgG peroxidase conjugate (Sigma) was used as secondary antibody at a final concentration of 70 μ g/L. Western blot analysis is consistent with the notion that monoclonal anti-K8Bio H4 does not cross-react with nonbiotinylated histone H4 and biotinylated histones H1, H2A, H2B and H3, but specifically binds to biotinylated histone H4 (Fig. 1A).

Effectiveness of antibodies in ChIP assays was tested by using Western blot analysis. Chromatin was precipitated by using polyclonal anti-K12Bio H4 as described above for ChIP assays; cross-linking with formaldehyde and digestion with protease were omitted. Proteins were resolved by gel electrophoresis as described [8] and transblots were probed by using the above antibody to the C-terminus in histone H4 (0.5 mg/L final concentration). Mouse monoclonal anti-rabbit IgG peroxidase conjugate (Sigma) was used as secondary antibody. Controls included transblots probed without primary antibody (negative control) and bulk extracts of Jurkat cell histones probed with the antibody to the C-terminus in histone H4 (positive control).

Finally, immunocytochemistry was used to determine whether monoclonal anti-K8Bio H4 colocalizes with biotinylated histones in the cell nucleus. Immunocytochemistry experiments were conducted in JAr human choriocarcinoma cells as described [9]. Primary antibody was used at a concentration of 3.3 mg/L. As secondary antibody we used Cy2-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA; 16 μ g/L final concentration). Cytoplasmic and nuclear compartments were stained with rhodamine phalloidin and 4',6-diamidino-2-phenylindole (DAPI), respectively, as described [24]. Images were obtained by using an Olympus FV500 confocal microscope (Microscopy Core Facility, University of Nebraska, Lincoln, NE). Immunocytochemistry data are consistent with the notion that monoclonal anti-K8Bio H4 colocalized with nuclear histones (Fig. 1B).

2.4. Semiquantitative PCR

Semiquantitative PCR was conducted as described [25] using the primers provided in Table 2 [26]. For hot-start PCR, we used Platinum TAQ DNA polymerase (Invitrogen, Carlsbad, CA) and equal amounts (micrograms) of immunoprecipitated DNA and input DNA. The reactions were run at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, with annealing for 30 s and extension at 72°C for 30 s. Ethidium-bromide-stained bands were quantified by gel densitometry. Only values from the exponential phase of PCR amplification (typically 19–30 cycles) were included in

Table 3

Relative enrichment of heterochromatin, transcriptionally repressed chromatin and euchromatin by ChIP assay in Jurkat cells

PCR target	Chromatin	Antibody for ChIP	Relative enrichment by ChIP
Chr1alpha	Heterochromatin	K12Bio H4	1.9±0.06 ^a
		K8Bio H4	1.4±0.2 ^b
		K9Me2 H3	1.8±0.1 ^a
		K4Me3 H3	1.0±0.05 ^c
Chr4alpha	Heterochromatin	K12bio H4	1.9±0.1 ^a
		K8Bio H4	1.2±0.06 ^b
		K9Me2 H3	2.1±0.1 ^c
		K4Me3 H3	0.8±0.06 ^d
γ-G globin	Repressed chromatin	K12Bio H4	1.3±0.06 ^a
		K8Bio H4	0.9±0.08 ^b
		K9Me2 H3	1.3±0.08 ^a
		K4Me3 H3	0.7±0.06 ^c
IL-2 (unstimulated)	Repressed chromatin	K12Bio H4	1.4±0.06 ^a
		K8Bio H4	1.0±0.06 ^b
		K9Me2 H3	1.5±0.1 ^a
		K4Me3 H3	1.0±0.1 ^b
IL-2 (stimulated)	Active chromatin	K12Bio H4	0.7±0.06 ^a
		K8Bio H4	1.0±0.05 ^b
		K9Me2 H3	0.8±0.1 ^a
		K4Me3 H3	1.5±0.02 ^c
ADH5	Euchromatin	K12Bio H4	0.9±0.1 ^a
		K8Bio H4	1.0±0.05 ^a
		K9Me2 H3	0.9±0.1 ^a
		K4Me3 H3	2.0±0.05 ^b
GAPDH	Euchromatin	K12Bio H4	1.1±0.1 ^a
		K8Bio H4	1.4±0.1 ^b
		K9Me2 H3	1.1±0.05 ^a
		K4Me3 H3	2.2±0.2 ^c

Chromatin was immunoprecipitated by using antibodies to K12Bio H4, K8Bio H4, K9Me2 H3 and K4Me3 H3. DNA was amplified by semiquantitative PCR for 19 to 30 cycles; only values from within the exponential phase of amplification were considered for quantitative analysis. Values represent the relative enrichment of precipitated DNA compared with 0.1% of input DNA (mean±S.D., $n=3$). Enrichment of interleukin-2 was quantified with and without stimulation of gene expression, using 50 µg/L of PMA and 2 mg/L of PHA for 2 h. ADH5=aldehyde dehydrogenase 5; Chr1alpha=chromosome 1 alpha satellite repeats; Chr4alpha=chromosome 4 alpha satellite repeats; IL-2=interleukin-2. ^{a,b,c,d}Rows not sharing the same letter are significantly different for a given PCR target ($P<.05$).

data analysis. Gel densitometry data from ChIP experiments represent three independent PCR reactions each conducted using DNA from independent immunoprecipitations. Note that it can be misleading to compare relative enrichments between ChIP experiments with different antibodies, which might have distinct efficiencies in ChIP assays.

We used reverse transcriptase PCR to confirm that transcription of heterochromatin was quantitatively minor compared with euchromatin. Transcripts from genes and alpha satellite repeats were detected as described [25], using the primers (denoted “Trans”) provided in Table 2. Theoretically, contamination of RNA with genomic DNA might produce false-positive results in PCR experiments. For the *interleukin-2* gene, this possibility was formally excluded by the size of the anticipated PCR product: 4655 bp if genomic DNA was the template vs. 390 bp if

complementary DNA was the template. For all other PCR reactions, we included controls in which genomic DNA was removed by treating RNA extracts with RNase-free DNase in combination with RNeasy spin columns (Qiagen).

2.5. Real-time PCR

For select genomic loci (alpha satellite repeats in chromosome 1, interleukin-2 promoter) we confirmed the relative enrichment in immunoprecipitated DNA by using real-time PCR (iCycler IQ multicolor real-time detection system; Bio-Rad, Palo Alto, CA). For amplification of alpha satellite sequences we used the same primers that were used for semiquantitative PCR; for interleukin-2 promoter sequences we used a pair of primers distinct from that used for semiquantitative PCR (Table 2). Moreover, the abundance of mRNA coding for HCS in siRNA-treated cells was quantified by real-time PCR. Absolute QPCR SYBR Green fluorescein mix (ABgene, Rochester, NY) was used for real-time PCR reactions.

2.6. Statistical analysis

Homogeneity of variances among groups was confirmed by using Bartlett’s test [27]. Significance of differences among groups was tested by one-way ANOVA. Fisher’s Protected Least Significant Difference procedure was used for post hoc testing [27]. StatView 5.0.1 (SAS Institute, Cary, NC) was used to perform all calculations. Differences were considered significant if $P<.05$. Data are expressed as mean±S.D.

3. Results and discussion

Jurkat cell chromatin was precipitated by using antibodies against K12Bio H4, K8Bio H4, K9Me2 H3 and K4Me3 H3. In a first series of ChIP assays, the purified DNA was amplified by semiquantitative PCR, using primers specific for pericentromeric alpha satellite repeats in chromosomes 1 and 4 (Table 2). Precipitation of chromatin by using anti-K12Bio H4 produced a 1.9-fold enrichment of alpha satellite repeats from chromosomes 1 and 4, respectively, compared with input DNA (Fig. 2 and Table 3). The fold enrichment by anti-K12Bio H4 was very similar to the

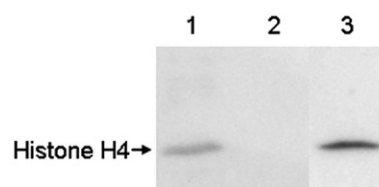


Fig. 3. The antibody to K12Bio H4 is effective in immunoprecipitations. Chromatin from Jurkat cells was immunoprecipitated by using antibody to K12Bio H4, and proteins were resolved by gel electrophoresis. Transblots were probed with an antibody to the C-terminus in histone H4 (Lane 1) or in the absence of primary antibody (Lane 2). Bulk histone extracts from Jurkat cells were probed with the antibody to the C-terminus in histone H4 and served as positive control (Lane 3).

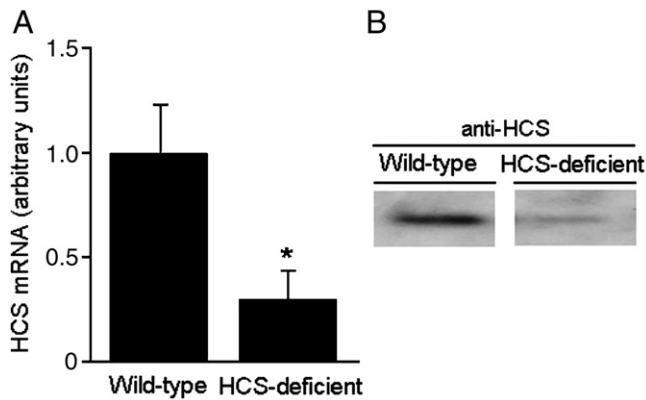


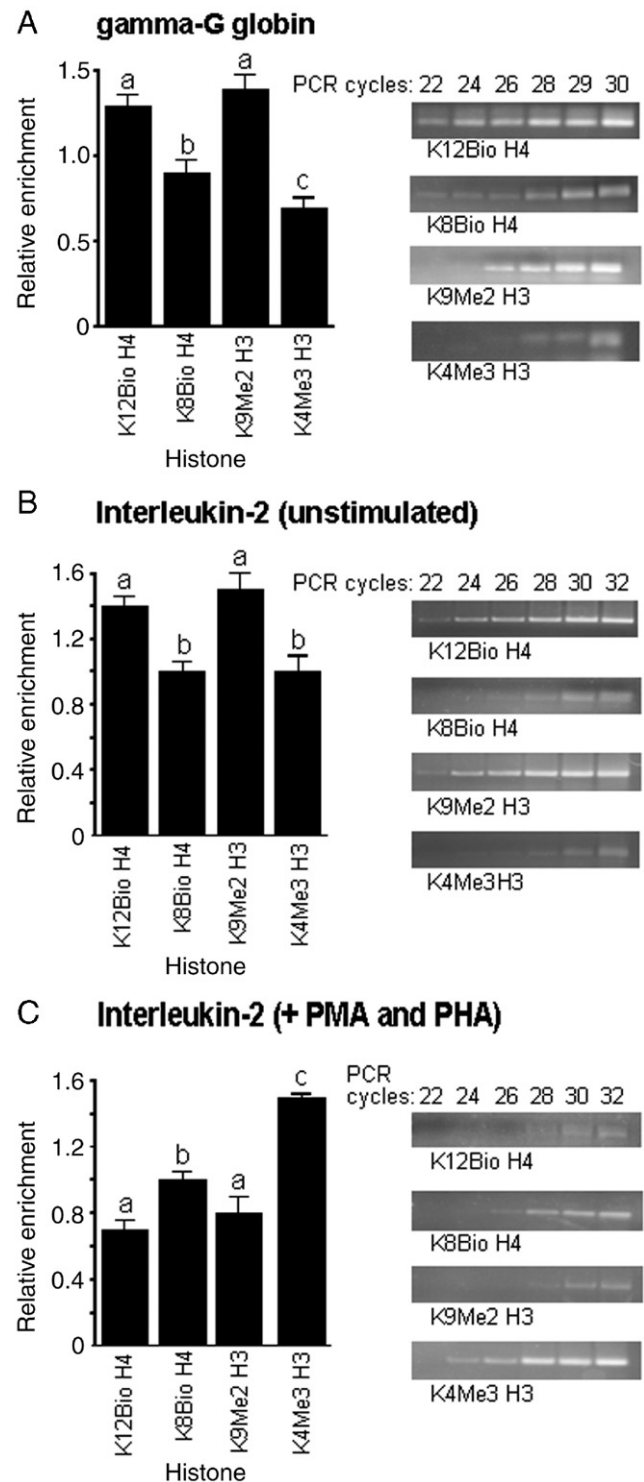
Fig. 4. Expression of HCS decreases in response to transformation of Jurkat cells with HCS-specific siRNA. Expression of HCS was quantified by real-time PCR (A) and Western blot analysis (B) in cells treated with an empty siRNA vector (denoted “Wild-type”) and cells treated with an HCS-specific vector (denoted “HCS-deficient”). Values are means \pm S.D. (* $P < .05$ vs. wild-type controls, $n = 3$).

1.8- to 2.1-fold enrichment observed for the known heterochromatin mark K9Me2 H3, suggesting that K12Bio H4 is associated with heterochromatin. In contrast, ChIP with an antibody to the euchromatin marker K4Me3 H3 was not associated with enrichment of alpha satellite repeats. Finally, precipitation of chromatin by using anti-K8Bio H4 produced a 1.2- and 1.4-fold enrichment of alpha satellite repeats from chromosomes 1 and 4, respectively, compared with input DNA. This level of enrichment is intermediate relative to that observed for anti-K9Me2 H3 (marker for heterochromatin) and anti-K4Me3 H3 (marker for euchromatin). More importantly, the levels of enrichment observed here are similar to levels reported in previous studies in humans and mice [5–7]. Collectively, these observations are consistent with the notion that K12Bio H4 and, perhaps, K8Bio H4 play roles in heterochromatic structures.

We conducted the following experiments to demonstrate that the above observations were specific for K8Bio H4 and K12Bio H4. First, chromatin was precipitated with bulk rabbit IgG; no enrichment of alpha satellite repeats was observed in these experiments (data not shown). Second, chromatin was immunoprecipitated with anti-K12Bio H4 and proteins were resolved by gel electrophoresis. Immunoprecipitation specifically enriched for histone H4, as judged by probing with an antibody to the C-terminus in histone H4 (Fig. 3, Lane 1). No bands were detectable if

Fig. 5. Relative enrichment of transcriptionally repressed chromatin by ChIP assay in Jurkat cells. Chromatin was immunoprecipitated using antibodies to K12Bio H4, K8Bio H4, K9Me2 H3 and K4Me3 H3. DNA was amplified by semiquantitative PCR, using primers specific for the promoter regions in genes coding for γ -G globin (A) and interleukin-2 (B); enrichment for interleukin-2 was also quantified after stimulation of interleukin-2 expression with 50 μ g/L of PMA and 2 mg/L of PHA for 2 h (C). Only values from within the exponential phase of amplification were considered for quantitative analysis. Bar graphs depict the relative enrichment of precipitated DNA compared with input DNA (mean \pm S.D., $n = 3$). ^{a,b,c} Bars not sharing the same letter are significantly different ($P < .05$). Gels depict representative examples from PCR.

transblots were probed with secondary antibody in the absence of primary antibody (Lane 2). Bulk extracts of Jurkat cell histones served as positive control and produced a strong signal (Lane 3). Third, HCS-deficient Jurkat cells were generated by using siRNA. The abundance of mRNA coding for HCS decreased by $70.5 \pm 6.6\%$ in siRNA-treated cells compared with wild-type controls (Fig. 4A); in contrast, the abundance of mRNA coding for GAPDH



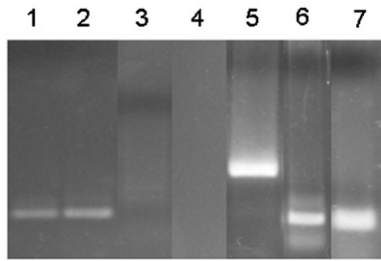


Fig. 6. Abundance of transcripts in Jurkat cells. Transcripts from the following regions in chromatin were amplified by RT-PCR: alpha satellite repeats on chromosomes 1 and 4 (Lanes 1 and 2, respectively), γ -G globin (Lane 3), interleukin-2 in unstimulated cells (Lane 4), interleukin-2 in cells stimulated with 50 μ g/L of PMA and 2 mg/L of PHA for 2 h (Lane 5), aldehyde dehydrogenase 5 (Lane 6) and GAPDH (Lane 7). Samples were amplified for 32 PCR cycles.

was not affected by knockdown of HCS, suggesting specificity of treatment (data not shown). Decreased abundance of HCS mRNA was associated with decreased abundance of the corresponding protein (Fig. 4B), as judged by gel densitometric analysis of Western blots (arbitrary units): 7.2 ± 0.3 units in wild-type cells vs. 1.2 ± 0.1 units in siRNA-treated cells ($n=3$; $P<0.05$). The control protein histone H3 was not affected by transformation with the siRNA vector (data not shown). The abundance of biotinylated histones was substantially decreased in HCS-deficient cells. For example, if chromatin from knockdown cells was precipitated with anti-K12Bio H4, the amount of DNA was only about 10% compared with wild-type controls. Fourth, real-time PCR (as opposed to semiquantitative PCR) was used to quantify relative enrichment of alpha satellite repeats in chromosome 1. Here, we used a monoclonal antibody to K8Bio H4 to independently confirm the observations made with the polyclonal antibody to K12Bio H4 in the experiments above; in addition, we used polyclonal anti-K12Bio H4 to quantify the relative enrichment of repeat sequences by real-time PCR. Briefly, the relative enrichment of alpha satellite repeats was 1.4 ± 0.04 for K12Bio H4, 1.5 ± 0.04 for K8Bio H4 and 1.4 ± 0.05 for K9Me2 H4 ($P<0.05$). These trends are similar to those described above.

Biotinylated histones H4 were also associated with transcriptionally repressed chromatin, following a pattern similar to that observed for alpha satellite repeats. Immunoprecipitation of chromatin by using anti-K12Bio H4 caused a 1.3- and 1.4-fold enrichment of γ -G globin and interleukin-2 sequences, respectively, as judged by semiquantitative PCR (Fig. 5 and Table 3). The fold enrichment by anti-K12Bio H4 was similar to the 1.3- to 2.1-fold enrichment observed by using anti-K9Me2 H3, suggesting that K12Bio H4 is not only associated with pericentromeric repeats but also with transcriptionally repressed chromatin. Enrichment of γ -G globin and interleukin-2 sequences by ChIP with anti-K8Bio H4 was weaker than that observed for anti-K12Bio H4. The relative enrichments at the interleukin-2 promoter locus were confirmed by real-time PCR. The

following enrichments were observed: 1.5 ± 0.2 for K12Bio H4, 1.7 ± 0.2 for K9Me2Bio H3 and 1.2 ± 0.1 for K4Me3Bio H3 ($P<0.05$). These levels of enrichment are similar to those calculated in experiments using semiquantitative PCR.

We used reverse transcriptase PCR to confirm that the transcription of repressed chromatin (γ -G globin and interleukin-2) was quantitatively minor compared with transcriptionally active chromatin (Fig. 6). Note that transcripts of alpha satellite repeats were detectable by reverse transcriptase PCR (Fig. 6), which is consistent with previous observations [5]. In control experiments, RNA was treated with DNase before reverse transcriptase PCR to remove any genomic DNA. Treatment with DNase did not decrease the signal intensity obtained by reverse transcriptase PCR (data not shown), suggesting that PCR products originated from RNA.

K12Bio H4 localized specifically to the transcriptional start site of the interleukin-2 gene as opposed to localizing

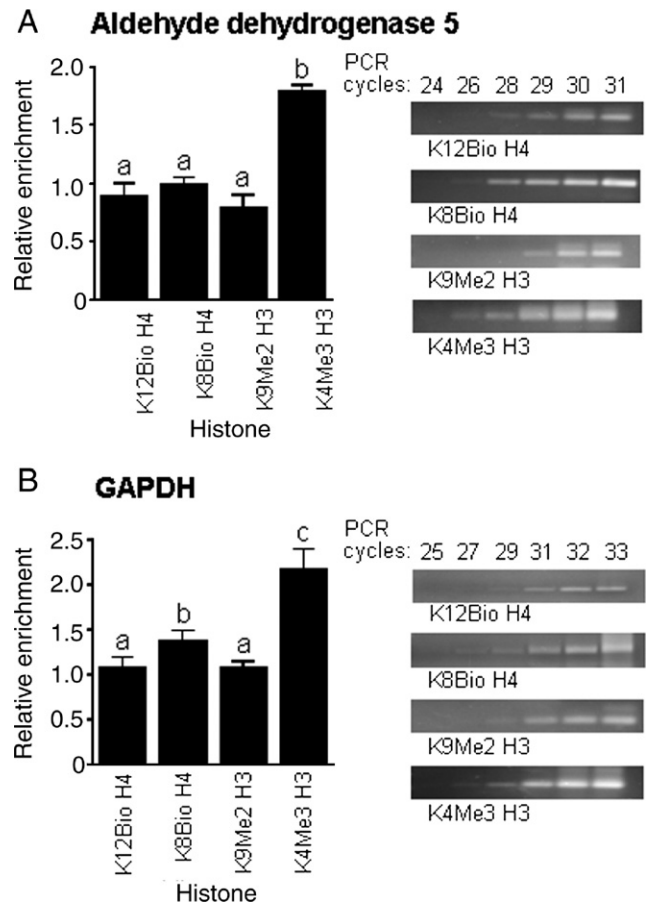


Fig. 7. Relative enrichment of euchromatin by ChIP assay in Jurkat cells. Chromatin was immunoprecipitated by using antibodies to K12Bio H4, K8Bio H4, K9Me2 H3 and K4Me3 H3. DNA was amplified by semiquantitative PCR, using primers specific for aldehyde dehydrogenase 5 (A) and GAPDH (B); only values from within the exponential phase of amplification were considered for quantitative analysis. Bar graphs depict the relative enrichment of precipitated DNA compared with input DNA (mean \pm S.D., $n = 3$). ^{a,b,c} Bars not sharing the same letter are significantly different ($P<0.05$). Gels depict representative examples from PCR.

equally to the entire gene locus. Here, we quantified the relative enrichment of sequences from the promoter region, the 5' end and the 3' end of the *interleukin-2* gene by using anti-K12Bio H4 and semiquantitative PCR (see Table 2 for PCR primers). Sequences from the promoter region of the *interleukin-2* gene were enriched 1.5 ± 0.3 -fold by immunoprecipitation with anti-K12Bio H4, confirming the data from Fig. 5. Likewise, sequences from the 5' end region of the *interleukin-2* gene were enriched 1.4 ± 0.1 -fold by using anti-K12Bio H4. In contrast, sequences from the 3' end of the *interleukin-2* gene were enriched only 0.9 ± 0.4 -fold by using anti-K12Bio H4. Previous studies have suggested that the majority of histone modifications involved in gene regulation reside within -500 to $+500$ bp of the transcription start site [6]. Collectively, these observations are consistent with the notion that K12Bio H4 participates in the regulation of transcriptional activity.

Next, we determined whether stimulation of interleukin-2 expression by treatment with PMA and PHA was associated with corresponding changes of histone biotinylation at the *interleukin-2* promoter. Interleukin-2 was selected as a model because expression of this cytokine is below the limit of detection in unstimulated lymphoid cells and increases substantially in response to mitogenic stimulation, as judged by using mRNA abundance, reporter gene activity and secretion of interleukin-2 into culture medium [28,29]. Here, we observed a substantial decrease in the relative enrichment of *interleukin-2* promoter sequences in chromatin from stimulated Jurkat cells precipitated with anti-K12Bio H4 compared with unstimulated controls (Fig. 5, compare B and C, and Table 3): enrichment decreased from about 1.4-fold in unstimulated cells to 0.7-fold in stimulated cells. Likewise, enrichment decreased by about 50% if chromatin from stimulated cells was precipitated using anti-K9Me2 H3 compared with unstimulated controls. Treatment with PMA and PHA did not affect the relative enrichment of interleukin-2 promoter sequences by anti-K8Bio H4. A potential explanation for this absence of effect has been offered above. Also, treatment with PMA and PHA did not affect the relative enrichment of *interleukin-2* promoter sequences by an antibody to K12-acetylated histone H4 (K12Ac H4; data not shown). K12ac H4 is a marker for transcriptionally active chromatin [2,3]; it remains uncertain as to why mitogenic stimulation of Jurkat cells was not associated with increased K12 acetylation at the interleukin-2 locus in this study. Treatment of Jurkat cells with PMA and PHA was associated with a 50% increase in the enrichment of *interleukin-2* promoter sequences in cells precipitated with anti-K4Me3 H3 (positive control). Reductions of K12Bio H4 and K9Me3 H3 levels in response to gene activation were apparently not caused by nucleosomal depletion at the *interleukin-2* locus. To independently verify changes in nucleosomal abundance, we utilized antibodies to the C-terminus of histone H4. This region of the H4 molecule is not a known target for posttranslational modifications

[2,3] and an antibody to this portion of the H4 protein should be expected to immunoprecipitate all chromatin containing bound and accessible histone H4. When chromatin was precipitated with such an antibody, no differences in the relative abundance of interleukin-2 promoter sequences were observed between stimulated cells compared with unstimulated controls (primer denoted "IL-2prom" in Table 2; data not shown). These results, in combination with the data obtained by using biotinylation-specific antibodies, collectively suggest that biotinylated histone H4 is not only associated with heterochromatin but also with transcriptionally repressed chromatin. Moreover, transcriptional activation of repressed chromatin genes appears to be associated with a debiotinylation of K12Bio H4 in the promoter regions of genes.

Consistent with the observations described above, antibodies to K12Bio H4 and K8Bio H4 did not enrich for aldehyde dehydrogenase 5 and GAPDH (Fig. 7 and Table 3). Also, no enrichment of these sequences was observed if chromatin was precipitated by using anti-K9Me2 H3. In contrast, precipitation of chromatin by using anti-K4Me3 H3 was associated with a 2.0- and 2.2-fold enrichment of promoter sequences from aldehyde dehydrogenase 5 and GAPDH, respectively.

Collectively, the study presented here provides evidence for a potential role of K12Bio H4 and, perhaps, K8Bio H4 in centromeric heterochromatin and silencing of euchromatin. These findings are consistent with the following observations, suggesting a role for biotinylation of histone H4 in chromatin condensation. First, biotinylation of both K8 and K12 in histone H4 increases during chromatin condensation in the M phase of the human cell cycle [15]. Second, biotinylation of K12 in histone H4 decreases transiently for about 30 min after occurrence of DNA double-strand breaks, but returns to initial levels about 1 h after induction of DNA breaks [14]. This is consistent with chromatin decondensation immediately following DNA breaks to give repair proteins access to sites of damage, followed by condensation of chromatin to prevent dissociation of broken ends [30]. The observations reported here have potentially far-reaching implications for biotin nutrition in human health. For example, about one third of pregnant women are marginally biotin deficient [31], with the effects on gene silencing, cell proliferation and DNA being unknown.

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