UNIVERSITY OF BIRMINGHAM
University of Birmingham Research at Birmingham

Developmental activation of the lysozyme gene in chicken macrophage cells is linked to core histone acetylation at its enhancer elements

Myers, FA; Lefevre, P; Mantouvalou, E; Bruce, K; Lacroix, C; Bonifer, Constanze; Thorne, AW; Crane-Robinson, C

DOI:
10.1093/nar/gkl543

License:
Creative Commons: Attribution-NonCommercial (CC BY-NC)

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (Harvard):
Myers, FA, Lefevre, P, Mantouvalou, E, Bruce, K, Lacroix, C, Bonifer, C, Thorne, AW & Crane-Robinson, C 2006, 'Developmental activation of the lysozyme gene in chicken macrophage cells is linked to core histone acetylation at its enhancer elements', *Nucleic Acids Research*, vol. 34, no. 14, pp. 4025-4035. https://doi.org/10.1093/nar/gkl543

Link to publication on Research at Birmingham portal

Publisher Rights Statement:
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.0/uk/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.
Copyright 2006 The Author(s).
Eligibility for repository: checked 01/06/2015

General rights
Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

• Users may freely distribute the URL that is used to identify this publication.
• Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
• User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
• Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy
While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

Download date: 27. Sep. 2020

Published online 16 August 2006 Nucleic Acids Research, 2006, Vol. 34, No. 14 4025–4035
doi:10.1093/nar/gkl543

Developmental activation of the lysozyme gene in chicken macrophage cells is linked to core histone acetylation at its enhancer elements

Fiona A. Myers, Pascal Lefevre[1], Evangelia Mantouvalou, Kimberley Bruce, Claire Lacroix[1], Constanze Bonifer[1], Alan W. Thorne and Colyn Crane-Robinson*

Biophysics Laboratories, Institute of Biomedical and Biomolecular Sciences, Faculty of Science, University of Portsmouth, Portsmouth PO1 2DT, UK and [1]Molecular Medicine Unit, St James's University Hospital, University of Leeds, Leeds LS9 7TF, UK

Received June 7, 2006; Revised July 5, 2006; Accepted July 7, 2006

ABSTRACT

Native chromatin IP assays were used to define changes in core histone acetylation at the lysozyme locus during developmental maturation of chicken macrophages and stimulation to high-level expression by lipo-polysaccharide. In pluripotent precursors the lysozyme gene (Lys) is inactive and there is no acetylation of core histones at the gene, its promoter or at the upstream *cis*-control elements. In myeloblasts, where there is a very low level of Lys expression, H4 acetylation appears at the *cis*-control elements but not at the Lys gene or its promoter: neither H3 nor H2B become significantly acetylated in myeloblasts. In mature macrophages, Lys expression increases 5-fold: H4, H2B and H2A.Z are all acetylated at the *cis*-control elements but H3 remains unacetylated except at the −2.4 S silencer. Stimulation with LPS increases Lys expression a further 10-fold: this is accompanied by a rise in H3 acetylation throughout the *cis*-control elements; H4 and H2B acetylation remain substantial but acetylation at the Lys gene and its promoter remains low. Acetylation is thus concentrated at the *cis*-control elements, not at the Lys gene or its immediate promoter. H4 acetylation precedes H3 acetylation during development and H3 acetylation is most directly linked to high-level Lys expression.

INTRODUCTION

Development of pluripotent chicken myeloid progenitor cells through myeloblasts to mature macrophages is accompanied by upregulation of the lysozyme gene consequent upon a step-wise opening of the lysozyme locus and the binding of transcription factors during the process of cellular differentiation (1,2). The tissue-specific Lys gene is located within a short locus of ~24 kb of chromatin that contains all the elements required for position-independent and tissue-specific expression of the gene and displays enhanced general DNaseI sensitivity between two matrix attachment regions, MARs, (3–5). In addition to the Lys gene, the locus also includes the constitutively transcribed Gas41 gene (6) that codes for a component of the NuA4 complex (7). At least five *cis*-regulatory elements control activation of the Lys gene in macrophages: enhancers at 6.1, 3.9 and 2.7 kb upstream of the start site, a silencer at 2.4 kb (−2.4S) and a complex proximal promoter, all of which are marked by developmentally regulated DNaseI hypersensitive sites (DHS). Investigation of the roles played by the several elements marked by DHS in the generation of tissue-specific expression of the lysozyme gene, in particular using transgenic mice, has shown that all must be present to achieve position independence and tissue-specific copy number-dependent expression (3).

Investigation of this complex regulatory system has been aided by the availability of several retrovirally transformed cell lines that correspond well to stages of macrophage development. Those used here are as follows:

(i) *Erythroblast, HD37 cells*: These are AEV-transformed early erythroid, i.e. erythroblast-like, non-myeloid cells (8) which do not transcribe the Lys gene. The −2.4S silencer DHS, present in all lysozyme non-expressing and weakly expressing cells is open, disappearing only on stimulation of promacrophages (9): it is bound by the enhancer-blocking protein CTCF (10). None of the other DHS is open however. Expression of the housekeeping Gas41 gene is higher than in the myeloid cells (Figure 1).

(ii) *Multipotent erythroid progenitor 50, MEP50 cells*: These correspond to myeloblasts and are AMV-transformed pluripotent myeloid precursors (8) in which the Lys gene is not transcribed (Figure 1), the 'early' DHS at the upstream −6.1E and −3.9E

*To whom correspondence should be addressed. Tel: +44 23 92842055; Fax: +44 23 92842053; Email: colyn.crane-robinsin@port.ac.uk

© 2006 The Author(s).
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.0/uk/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Downloaded from http://nar.oxfordjournals.org/ at University of Birmingham on June 1, 2015

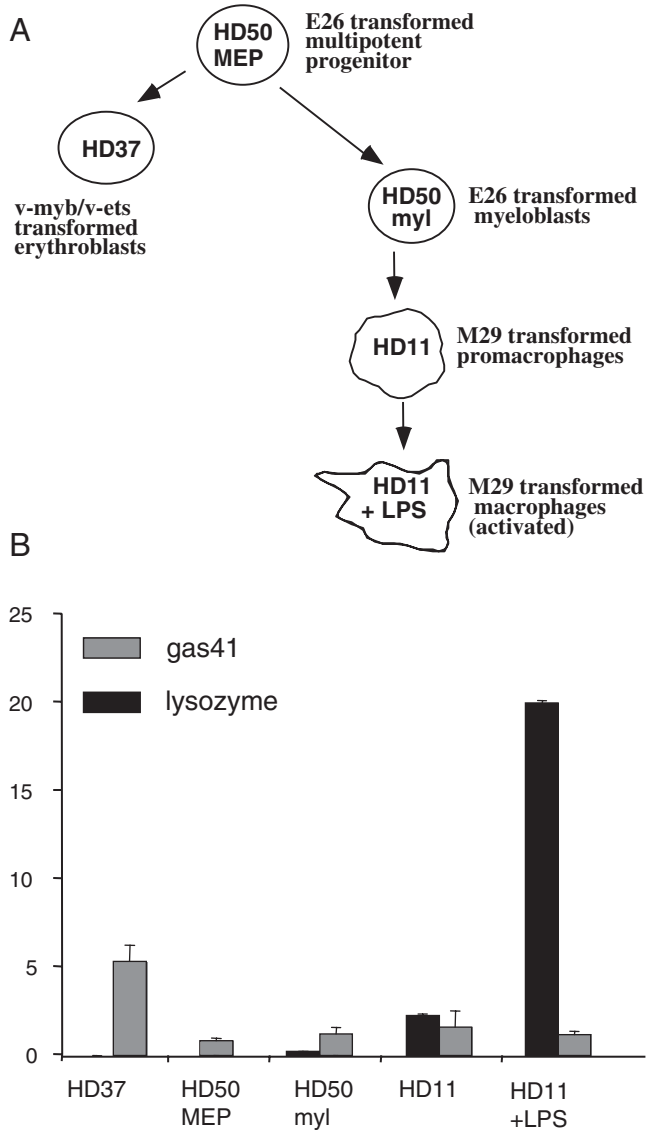

Figure 1. (**A**) The cell lines used and their relationship. (**B**) Relative mRNA levels of Gas41 and Lysozyme in the cell lines used here.

enhancers and the gene promoter are absent but the −2.4S silencer DHS is open. In comparison with HD37 erythroblast cells, it was shown by photo-footprinting that the chromatin of the locus has begun to open in MEP50 cells (11). Despite the absence of DHS, partial HinfI accessibility was found at the −6.1E and −3.9E enhancers in MEP50s and particularly in the region of −2.4S/−2.7E, presumably due to −2.4S activity, but not at the Lys promoter. Furthermore, nuclear factor 1 (NF1) transiently interacts with the upstream enhancers, but not with the promoter (2).

(iii) *Myeloblast, HD50myl cells*: These are E26-transformed myeloblast cells resembling granulocyte–macrophage precursors (8) in which DHS appear at both the −6.1E and −3.9E enhancers and at the promoter. The −2.4S silencer remains hypersensitive but there is only a very low level of Lys transcription (9). Investigation of the chromatin structure showed increased HinfI accessibility at both −6.1E and −3.9E (as compared with MEP50), as well as in the −2.4S/−2.7E region, demonstrating further opening of the locus: additionally, despite the very low level of transcription (Figure 1), high HinfI accessibility is also seen at −52 bp in the proximal Lys promoter (11).

(iv) *HD11 promacrophage cells*: These are MC29-transformed promacrophages (12) in which there is an increased level of lysozyme expression (Figure 1). The DHS at the −6.2E and −3.9E enhancers and the −2.4S silencer remain and additionally a 'late' enhancer DHS appears at −2.7E, as well as two DHS at the proximal promoter of the Lys gene. LPS activation of HD11 promacrophage cells results in upregulation of Lys transcription a further 10-fold (Figure 1).

Core histone acetylation has been studied intensively at other multi-gene loci such as chicken, mouse and human β-globins in erythroid cells and the human growth hormone locus in pituitary and placenta (13–17). Under these circumstances H3 and H4 acetylation is typically widespread ('global') but no consistent pattern has emerged as regards the distribution between the more remote control elements and genes/proximal promoters. In cases where individual housekeeping and tissue-specific genes have been mapped, (rather than several genes clustered into specific loci), both H4 and H3 acetylation have been found to concentrate close to the transcriptional start sites and extend a short distance into the body of the gene (18–20). The aim of the present study is to map core histone acetylation at high resolution throughout the chicken lysozyme locus in order to establish links between these modifications and factor binding (11) and with other epigenetic marks, in particular methylation of H3 and DNA (21,22) and with structural features of the locus.

MATERIALS AND METHODS

Cell culture

The chicken monocytic HD11 cell line (12) and the HD50 MEP (MEP50), HD50myl and HD37 cell lines (8) were grown in DMEM containing 8% feotal calf serum, 2% chicken serum, 75 μg/ml conalbumin (Sigma), 0.03 U/ml insulin, 10^{-4}M β-mercaptoethanol, 100 U/ml of penicillin and 100 μg/ml of streptomycin. Where indicated the HD11 cells were stimulated with 5 μg/ml of LPS (Sigma) for 4 h.

Preparation of nucleosomes and their immunoselection (nChIP)

Following cell harvesting, nuclei were immediately prepared and treated with micrococcal nuclease under conditions that yielded a mixture of mono-, di and some tri-nucleosomes. These were subjected to histone H1 depletion and then separated on exponential sucrose gradients to yield mononucleosomes (13,23,24). They were then immunoselected with affinity purified polyclonal antibodies to hyperacetylated H4, H3, H2B and H2A.Z, and to unmodified H2A.Z (17,19,20). Antibody-bound nucleosomes were retained on protein A–Sepharose for rabbit antibodies (anti-hyperacetylated H4, H3 and H2B) and protein G–agarose beads for sheep antibodies (anti H2A.Z and hyperacetylated

Downloaded from http://nar.oxfordjournals.org/ at University of Birmingham on June 1, 2015

H2A.Z) and removed by centrifugal filtration through Spin-X filters. Unbound mononucleosomes were recovered in the filtrate. DNA and proteins were extracted from the immobilized bound immunocomplexes, the input and the unbound mononucleosomes. The proteins were visualized on acetic acid/urea/Triton (AUT) gels to check the effectiveness of the immunoselection process and the DNA was ethanol precipitated and UV quantified (23–25).

DNA sequence quantification

The purified DNA from the input, unbound and bound nucleosomes, together with chicken genomic DNA as standard, were analysed for sequence content by quantitative real-time PCR with Taqman probes on an ABI Prism 7900 (20). The amplicon lengths varied between 68 and 113 bp (primer and Taqman sequences available on request) and the results for each amplicon were expressed as the ratio **B/I** (which represents the absolute fold enrichment achieved by the ChIP) or, where a depletion occurred, as **I/B** (which represents the absolute fold depletion), all as described in (19). Error bars represent standard deviations of triplicate PCR measurements. To assess repeatability, ChIPs were carried out using hyperacetylated H4 antibodies with mononucleosomes generated from separate cultures of (untreated) HD11 cells: these gave enrichments/depletions that exhibited a root mean square variation of ±8%, i.e. the pattern of **B/I** values across the locus was very similar in independent experiments. Figures 3–6 give plots from single ChIPs and the **B/I** values are given in tabular form in Supplementary Tables T1–T4. The enrichment scale in the figures has been maintained constant for an individual antibody but since the five different antibodies are not equally effective, their enrichment scales differ, so that comparison of, say, H4 and H3 enrichments in a given cell type does not accurately represent relative levels of acetylation.

The validity of the observed enrichments/depletions

When equating observed enrichments (**B/I** values) to acetylation levels at a defined amplicon, it is essential to be sure that the nucleosome being interrogated is adequately represented in the input fraction. Comparison was therefore made for each preparation of mononucleosomes of the signal from the input DNA at each amplicon relative to that of an equal weight of sonicated genomic DNA (**G**). The **I/G** ratio was typically ~0.5, (a value less than unity owing to the differing DNA sizes) and any digest for which the **I/G** ratios fell substantially below this level was rejected.

RT–PCR

Following RNA extraction with TRIzol and first strand cDNA synthesis with oligo-dT primed Moloney murine leukaemia virus polymerase, real-time quantitative PCR was used on an ABI Prism 7700: all exactly as described in (22).

RESULTS

Antibodies used in ChIP experiments

All the antibodies used have been described previously. Antibodies to hyperacetylated H4 (19,25) and H2B (20) were raised against chemically acetylated forms of the histones and affinity purified against either the immunogen (AcH4) or a tetra-acetylated N-terminal peptide (AcH2B). Antibodies to hyperacetylated H3 (19) and H2A.Z (17) were raised against a tetra-acetylated (AcH3) or a tri-acetylated (AcH2A.Z) N-terminal peptide and affinity purified against the same peptides. Antibodies to unacetylated H2A.Z (17) were raised against an unmodified N-terminal peptide and affinity purified against the same peptide. AUT gel analysis of histones in the chromatin precipitated by these anti-hyperacetylated histone antibodies showed that they preferentially select the most highly acetylated species, though lower degrees of acetylation are also found in the immunoprecipitates. Substantial depletion of the unacetylated and monoacetylated species is observed in all cases, with the exception of AcH2B for which the immunoprecipitates contain considerable amounts of unmodified H2B co-existing with the tetra- and tri-acetylated forms: this suggests that in nucleosomes containing a single hyperacetylated H2B molecule, the other H2B remains unmodified.

As an example of the use of these antibodies with the present myeloid cells, Figure 2 shows an AUT gel of proteins immunoprecipitated from HD11 mononucleosomes with anti-hyperacetylated H4 antibodies. This shows not only the expected enrichment of hyperacetylated H4 species but

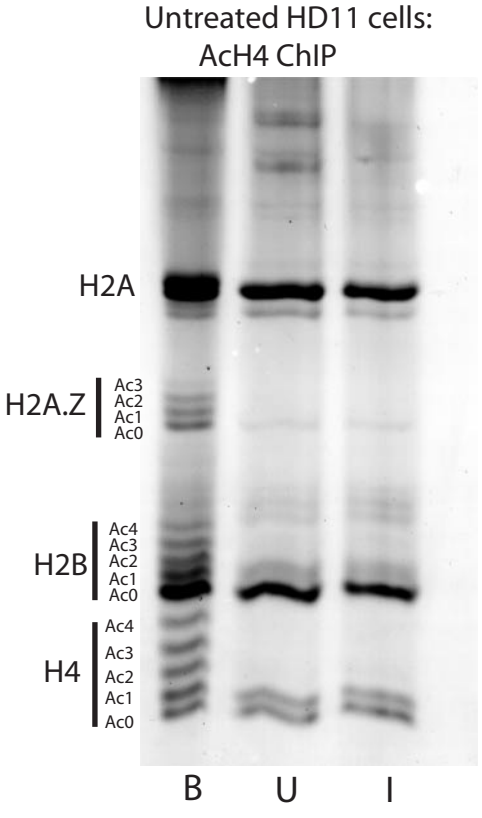

Figure 2. Proteins from an nChIP using mononucleosomes from HD11 cells and anti-hyperacetylated H4 antibodies, analysed on an acetic acid/urea/Triton (AUT) gel. **B**, bound fraction; **U**, unbound fraction; **I**, input fraction. Note that in addition to the expected enrichment of hyperacetylated species of H4, the Ab-bound nucleosomes are also enriched in acetylated species of H2B and also the replacement histone H2A.Z.

Downloaded from http://nar.oxfordjournals.org/ at University of Birmingham on June 1, 2015

co-enrichment of acetylated species of H2B and H2A.Z, an observation made previously with the same anti-hyperacetylated H4 antibodies (13,23). Importantly, the reciprocal observations have also been made: when immunoprecipitating with anti-hyperacetylated H2B antibodies, substantial enrichment of both hyperacetylated H4 and H2A.Z species was observed in the bound fractions [Figure 3 of (20)] and immunoprecipitations with anti-hyperacetylated H2A.Z antibodies led to substantial enrichments of both hyperacetylated H4 and H2B species [Figure 1 and Supplementary Data of (17)]. Since both the present and previous observations were made using fractionated mononucleosomes, the data demonstrate a high level of co-habitation, at the bulk level, of these three acetylated histones in the same nucleosomes. This seems also to be true for acetylated histone H3 since immunoprecipites using hyperacetylated H3 antibodies exhibited elevated levels of hyperacetylated H4 species and vice versa [Figure 2 of (19)]. The reciprocal observations for acetylated H3 have been harder to make, however, since the various sub-fractions of H3 run at multiple points on AUT gels.

This conclusion on the co-habitation of acetylated core histones would not be valid if any of the antibodies exhibited pan-acetyl activity, i.e. cross-reactivity to acetylated species of the other histones but their individual characterizations using SDS gels (17,19,20,25) make it clear that this is not the case. Observation of such 'bulk' co-habitation is not unexpected, however, in the light of 'genome-wide' mapping of acetylated H4, H3 and H2A.Z in *Saccharomyces cerevisiae* (26–29) and for H3 and H4 in *Drosophila melanogaster* (30) that show the acetylated histones congregating at the promoters and 5′ ends of active genes, a location demonstrated previously by individual gene mappings at high resolution (17–20). Bulk co-habitation of methylated histones in H2A.Z-containing nucleosomes has been investigated recently using mass spectrometry (rather than gels) of the histones following immuno-selection (31).

Histone H4 acetylation

Figure 3 shows that in HD37 cells there is virtually no acetylation of H4 across the upstream control region and the same is the case in MEP50 cells, despite the primed chromatin structure, not even at the open −2.4S DHS, or at the Lys gene or its promoter. In contrast, in both HD37 and MEP50 cells there is substantial H4 acetylation within the CpG island region of the Gas41 gene that encompasses the presumed promoter and 5′ transcribed region. This distribution is typical for a housekeeping gene (19,20). Corresponding studies of the methylation status of this region of the locus show that monomethyl H3/K9 is absent from the CpG island and levels of the activating tri-methyl H3/K4 are substantial (22). It is noteworthy that the immediate juxtaposition of the Lys and Gas41 genes (only 200 bp separates them) means that the acetylation extends to the highly enriched +3388 amplicon which is located in ExonIII of the Lys gene, 300 bp upstream of the Lys termination point. Halfway along the Gas41 gene, at the +5315 amplicon, H4 acetylation has fallen to extremely low levels. It is also seen from Figure 3 that within the two MARs there is no significant H4 acetylation, neither in HD37 nor MEP50 cells.

Development to the HD50 myeloblast stage results in the appearance of substantial H4 acetylation throughout the *cis*-control region, in particular at the open −2.4S silencer and −6.1E enhancer, less evidently at the −3.9E enhancer. Low levels are also seen at the 5′ end of the Lys gene but there is very little H4 acetylation at its proximal promoter: the CpG island region of the Gas41 gene retains H4 acetylation. Further development into the mature and adherent HD11 promacrophages leaves the overall distribution over the *cis*-control region largely unchanged, with the exception of a sharp increase in H4 acetylation at the −3.9E enhancer. When HD11 cells are treated with LPS and Lys expression rises a further 10-fold, H4 acetylation becomes widely distributed across the *cis*-control region. Significant acetylation becomes detectable at the Lys promoter and 5′ end of the transcribed region of the gene and the same is true of the constitutively expressed Gas41 gene.

Histone H3 acetylation

In both HD37 and MEP50 cells (Figure 4) H3 acetylation is, like H4 acetylation, essentially absent from the *cis*-control region. Surprisingly, this situation is largely retained in the HD50myl myeloblast cells, in contrast to H4 acetylation. Even in HD11 cells where Lys expression is quite significant (Figure 1), there is still very little H3 acetylation, with the sole exception of a hot spot at the −2.4S silencer. In this region of the locus, five very closely spaced amplicons were interrogated in a length of slightly <600 bp but only the nucleosome at −2447, that bears the CTCF and TR binding sites (32), showed a high enrichment. When however the HD11 cells were LPS treated, extensive H3 acetylation appeared throughout the *cis*-control region, the highest enrichment being just 3′ of the −2.4S silencer. These changes in H3 acetylation thus correlate with levels of Lys expression. In contrast, the Lys gene itself and its promoter lack H3 acetylation even in the LPS-stimulated HD11 cells. The 5′ end of the Gas41 gene maintains a low level of H3 acetylation throughout the developmental stages, the highest level being in the HD37 erythroblast cells where the level of Gas41 transcription is greatest (Figure 1). To confirm these unusual observations we also made use of an alternative differentiation system based on BM2 monoblast cells (22) that, untreated, resemble committed macrophage precursors, i.e. are somewhat more differentiated than HD50myl cells, monitoring acetylation of H3/K9. An increase in H3 acetylation was observed at the *cis* elements during BM2 differentiation and LPS stimulation, similar to that seen in HD11 cells, with a strong signal at −2.4S but little at the Lys promoter (data not shown).

Histone H2B acetylation

Figure 5 shows that this modification is not present at the Lys gene or its *cis*-control region either in HD37 or MEP50 cells and H2B acetylation does not appear in the myeloblast HD50myl cells, as for H3. Differentiation to HD11 macrophages, however, results in substantial H2B acetylation, particularly at the −6.1 kb enhancer and the region of the −2.4 kb silencer: on LPS treatment a similar distribution remains. H2B acetylation in the *cis*-control region thus seems

Downloaded from http://nar.oxfordjournals.org/ at University of Birmingham on June 1, 2015

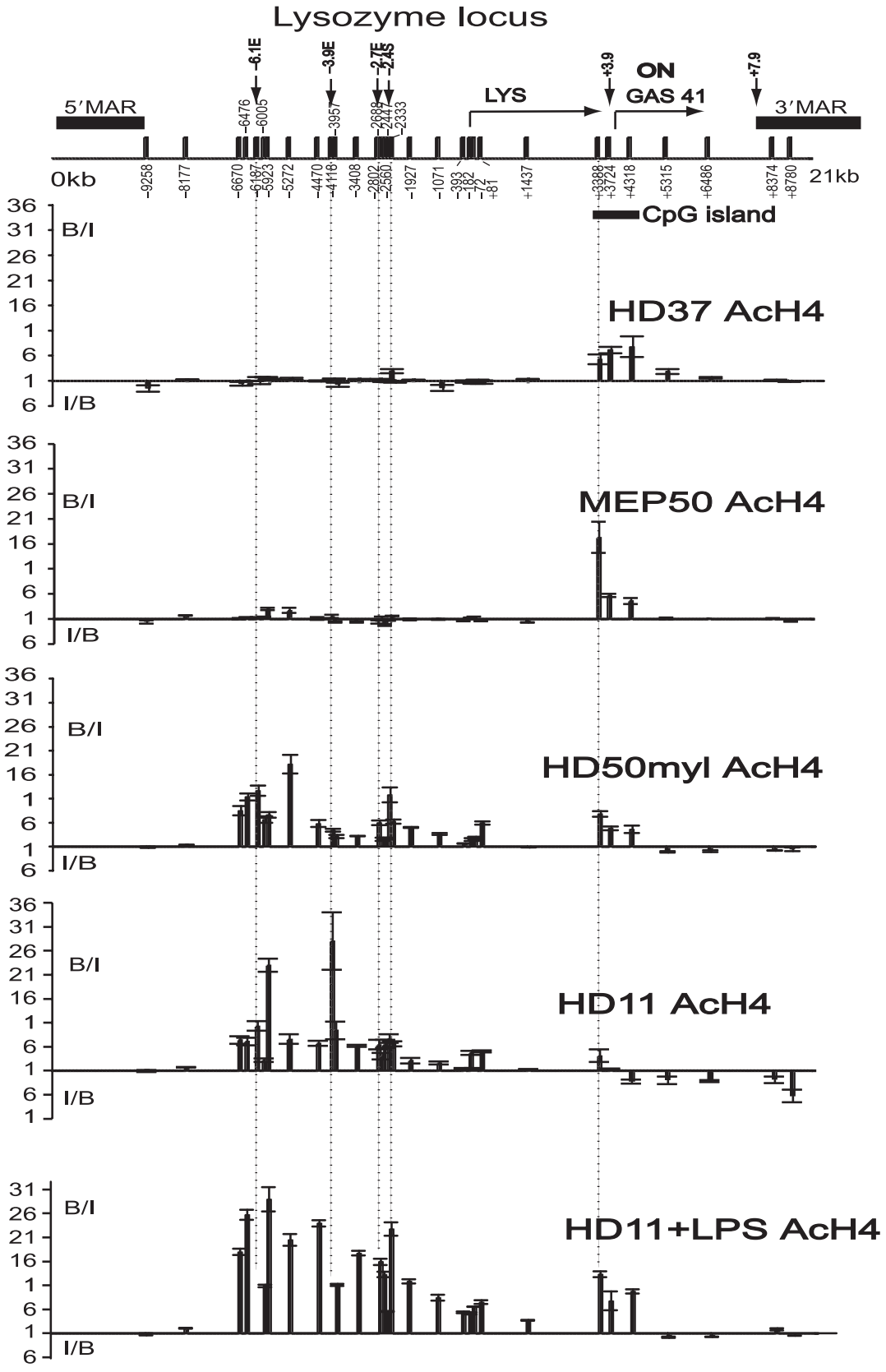

Figure 3. Distributions of hyperacetylated H4 at the lysozyme locus in the four cell types. Vertical arrows indicate DHS and vertical bars with numbers indicate amplicon positions numbered from +1 at the transcriptional start site. The two genes, their transcriptional status, the 5′ and 3′ MARs and the CpG island are indicated. **B/I** values (the ratio of the bound to the input signal for an amplicon) above the unity lines represent 'fold enrichment' achieved by the antibodies. **I/B** values are plotted below the unity lines when the bound signal is less than that of the input: this ratio represents 'fold depletion'.

Downloaded from http://nar.oxfordjournals.org/ at University of Birmingham on June 1, 2015

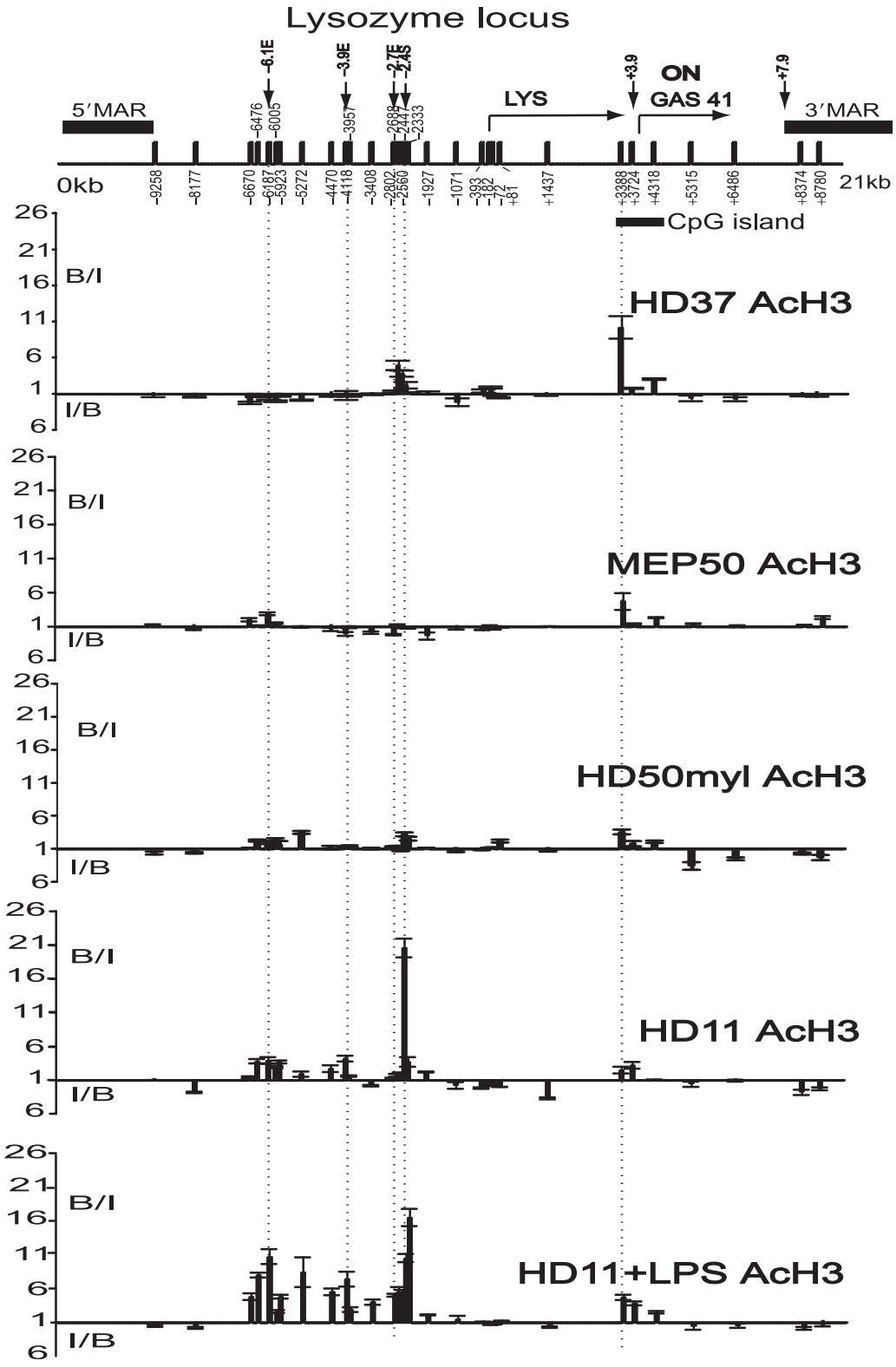

Figure 4. Distributions of hyperacetylated H3 at the lysozyme locus in the four cell types. Nomenclature as in Figure 3.

to be linked to actual transcription. No H2B acetylation whatsoever was observed at the Lys gene itself, though low levels were seen at the 5′ end of the Gas41 gene, especially in MEP50 and HD37 cells.

Histone H2A.Z acetylation

In HD37 erythroblasts (Figure 6) the acetylated form of the replacement histone H2A.Z was found to be highly enriched

Downloaded from http://nar.oxfordjournals.org/ at University of Birmingham on June 1, 2015

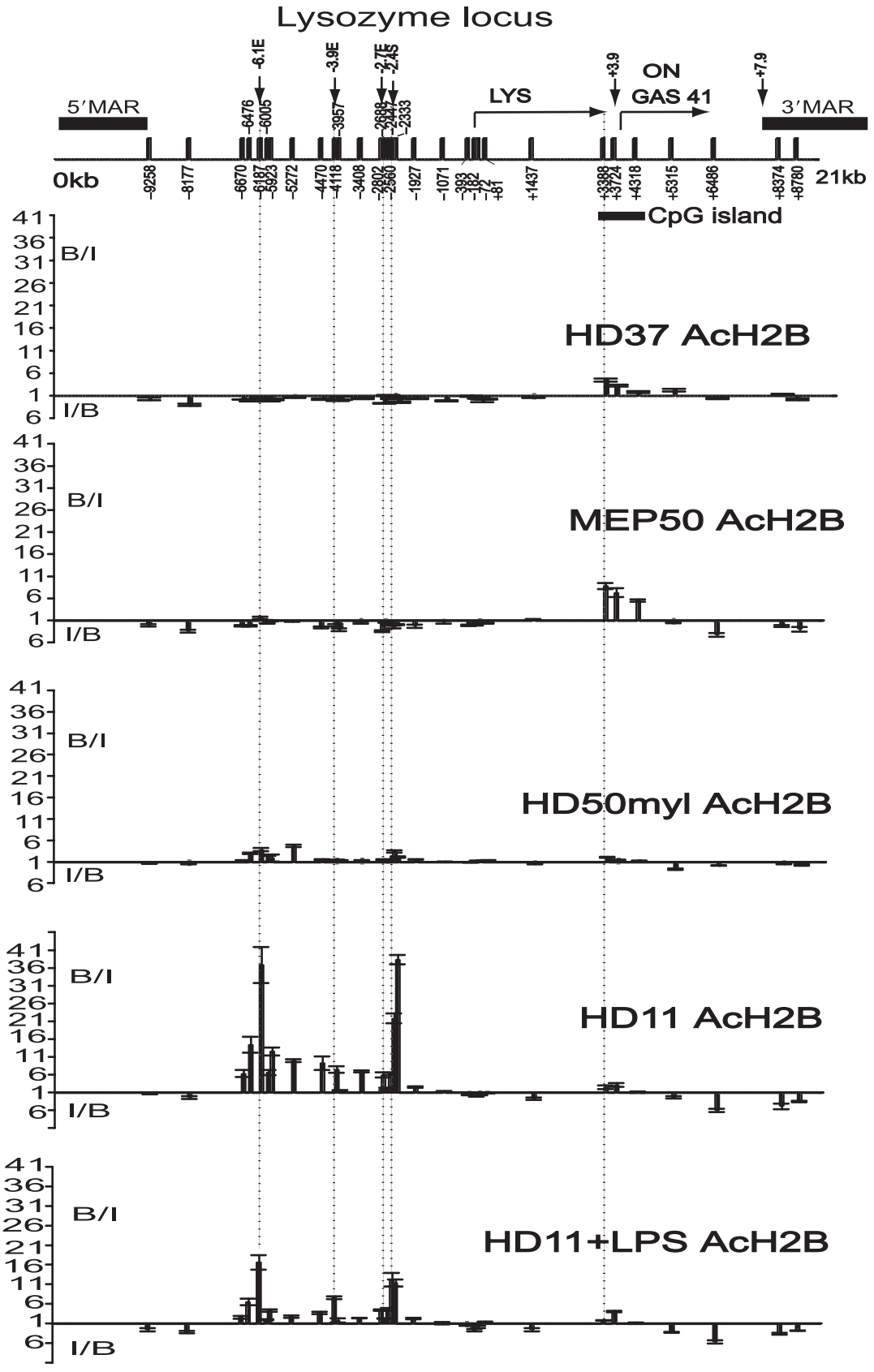

Figure 5. Distributions of hyperacetylated H2B at the lysozyme locus in the four cell types. Nomenclature as in Figure 3.

Downloaded from http://nar.oxfordjournals.org/ at University of Birmingham on June 1, 2015

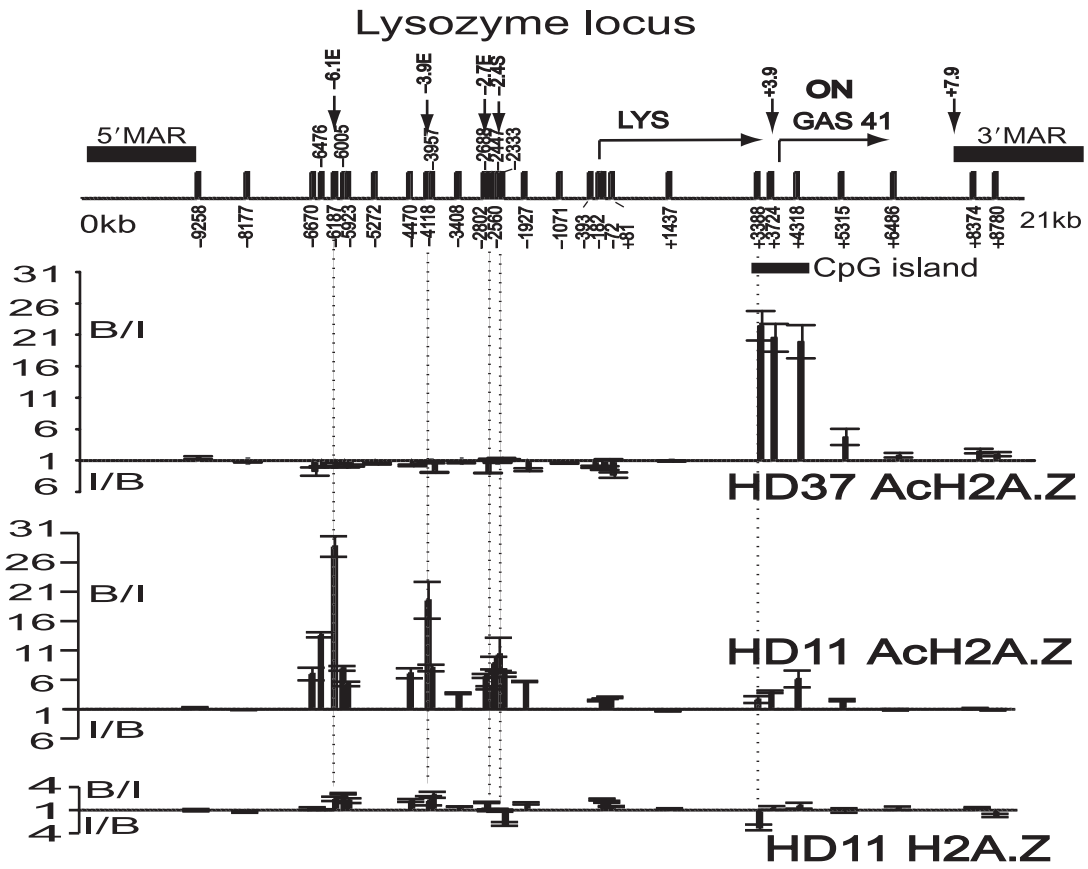

Figure 6. Distributions of hyperacetylated H2A.Z at the lysozyme locus in HD37 erythroblasts and untreated HD11 macrophages. The distribution, more precisely the absence, of unacetylated H2A.Z is shown at the bottom of the figure. Nomenclature as in Figure 3.

within the CpG island region of the Gas41 gene, as reported previously for other housekeeping genes (17), but is completely absent from the *cis*-control region of the Lys gene, as for the other core histones in HD37 cells. To test for association of H2A.Z with Lys transcription, ChIPs were performed with unstimulated HD11 cells. All three enhancer DHS (at −6.1, −3.9 and −2.7) and the −2.4S silencer were sites of substantial H2A.Z acetylation and in common with the other three core histones, levels at the Lys gene itself were low. There was no evidence for the unacetylated form anywhere in the locus. It follows that acetylation of H2A.Z is a feature not only of the proximal promoter and 5′-transcribed sequences of active genes (17) but also can be present in control regions, in this case up to ∼6 kb upstream of the start site.

DISCUSSION

Acetylation of the lysozyme locus in chicken macrophage cell lines

MEP50 progenitor cells show evidence of chromatin opening with transient binding of NF1 and partial demethylation of CpGs at the enhancers (12,21). The present observations show there is virtually no acetylation in MEP50 cells, i.e. initial opening of the *cis* elements is not dependent on core histone acetylation. When full commitment to the myeloid lineage has taken place (HD50myl) there is a just detectable level of Lys transcription. In the related BM2 monoblast cells, monomethyl H3/K9 has disappeared from the *cis* elements (except from the −2.4S silencer) and from the Lys promoter, and H3/K4 methylation has appeared at the *cis* elements (22). The present data show that in HD50myl myeloblasts, acetylation of histone H4 but not H3 is present at the *cis* elements. However, at the Lys gene itself and its promoter there are only low levels of H4 acetylation and no H3 acetylation.

Further development to HD11 cells involves the stable recruitment of the Ets family member Fli-1 to the open −6.1E and −3.9E enhancers. In addition, C/EBPβ becomes associated with all the enhancers and the proximal promoter. Upregulation of Lys is also accompanied by changes in histone methylation at the *cis*-control elements, in particular a rise in mono- and tri-methyl H3/K4 (22). The loss of K9 methylation is accompanied by a rise in acetylation of this residue but no increase in H3 acetylation at the promoter was noted however (2). Correspondingly, binding of CBP, a protein known to possess acetyltransferase activity towards histone H3, was shown to occur at the enhancers but not the promoter in HD11 cells. The present work shows that in unstimulated HD11s, the H4 acetylation at the *cis* elements is accompanied by that of H2B and by that of H3, but the last only at the −2.4S silencer.

LPS treatment of HD11 cells leads to a further increase in C/EBPβ binding that may represent a stabilization of the assembled transcription factor complexes (2) but no further

Downloaded from http://nar.oxfordjournals.org/ at University of Birmingham on June 1, 2015

recruitment of CBP (2). The present data show that on LPS activation of HD11 cells, H3 acetylation additionally appears throughout the *cis* elements but the Lys gene itself and its promoter still lack H3 acetylation despite the appearance of two promoter DHS and a substantial level of transcription. The acetylation of H3 at the *cis* elements thus appears important for the high-level expression that follows treatment with LPS.

The very low-level acetylation at the active Lys gene is in contrast to previously studied examples for which core histone acetylation, particularly of H3, at the promoter and 5′-transcribed sequences is observed, irrespective of whether the gene is isolated, such as chicken GAPDH (19), or together with other related and differentially expressed genes, such as Gas41 (above) and the chicken and mouse β-globin loci (14,15,20). There is substantial evidence that acetylation levels at promoters and transcribed sequences are directly related to levels of transcription. For example, a genome-wide assessment of histone H3 and H4 acetylation in *Drosophila* (30) found a correlation between the degree of modification for both histones and mRNA levels. In a study of adipocyte differentiation (33), monitoring in particular the *apM1* adiponectin gene, it was observed that the proximal promoter did not carry acetylated H3 in the primed precursor cells, i.e. in the 'poised' state, but it appeared on differentiation and the amount of AcH3 rose as the level of mRNA increased. By the time of maximal transcription (after 7 days of differentiation), H3 acetylation was also evident at a point about halfway along the 16 kb gene. Both the level and distribution of AcH3 thus appeared to correlate with rates of transcription. It might therefore be that only the very highest rates of transcription of the Lys gene, e.g. in the oviduct, require acetylation of the promoter and its transcribed region.

The finding that it is the *cis* elements rather than the active gene itself that carry the acetylated histones has also come to light in several recent large-scale mappings of H3 acetylation in the human genome: the majority was found to lie within ±500 bp of transcriptional starts, in particular at CpG islands, rather than in the body of the genes (34,35). Co-localization of acetylated H3 with upstream regulatory elements rather than the transcribed regions was also observed in a genome-wide study of human T-cells (36).

The present observations show that acetylation of H4 precedes that of H3: acetylation of H4 might therefore be critical for establishing transcriptional competence, whereas acetylation of H3 at the *cis* elements is directly linked to transcription itself. When acetylation of the *cis* elements first appears in HD50myl myeloblasts, it is predominantly of H4 but it is only on LPS treatment of HD11s that an intense H3 acetylation finally appears. Such a differential acetylation of H4 and H3 was also found during the viral induction of the human IFNβ gene, where acetylation of H4 at the promoter largely precedes the initiation of transcription and even falls to low levels when transcription is maximal: in contrast, H3 acetylation peaks at the time when IFNβ transcription starts and is maintained throughout the 12 h it continues (18). A more direct link of H3 rather than H4 acetylation to active transcription has also been demonstrated at the human β-globin locus: a fairly homogeneous pattern of H4 acetylation contrasts with the H3 acetylation that exhibits strong peaks at the LCR and at the active but not inactive globin genes (37). In a study of Ig class switching in mice it was found that association of RNA pol-II with S-regions correlated with acetylation of H3 rather than H4 (38). So far there have been no detailed large-scale (genome-wide) mappings of H4 acetylation of a similar type to those carried out for H3 that might, for example, link H4 acetylation to potentially active (poised) genes.

Other systems for which core histone acetylation has been monitored during differentiation include muscle development: myoblast differentiation into myotubes (39) and mouse T(H) cell differentiation (40). Naïve precursor T-cells do not carry H4 acetylation at the IFNγ gene characteristic of T_H1 cells, nor at the IL4 gene characteristic of T_H2 cells, a situation akin to that in the myeloid precursor MEP50s. After 8 days differentiation, H4 acetylation was observed at the promoter of the IFNγ gene in T_H1 cells and at the promoter, enhancer and two DHS of the IL4 gene in T_H2 cells, circumstances similar to the committed myeloblast HD50myl cells. Following stimulation by polarizing cytokines, the H4 acetylation at the four IL4 sites remained largely unchanged and acetylation of H3 appeared at the same sites, rather in the manner seen on LPS stimulation of HD11 cells. Since, however, no assay was made of H3 acetylation in the differentiated (but unstimulated) state of T_H2 cells, it is unfortunately impossible to judge whether H3 acetylation was subject to a 'temporal lag' relative to H4 acetylation in T_H2 cell development, in the manner observed here for macrophage development.

The involvement of H3 rather than H4 acetylation in transcription has been demonstrated very recently using active reconstituted templates bearing acetylated H3 and acetylated H4 histones to which chimeric repressor complexes containing HDAC activity were recruited. Repression of the H3-acetylated templates was completely dependent on the HDAC activity but this activity was not required to repress H4-acetylated templates (41).

A possible reason for the prior acetylation of H4 is structural: the N-terminal tail of this core histone appears to play a key role in maintaining chromatin higher order structure (in the absence of linker histone) and this is controlled by its acetylation state. An *in vitro* ultracentrifugation study of reconstituted polynucleosomal arrays assembled using recombinant N-terminally truncated core histones showed that the N-terminal tail of H4, particularly residues 14–19, but not that of the other three core histones was essential for formation of the compacted state (42). In a parallel study (43) it was shown that arrays assembled using H4 acetylated at K16 sedimented much more slowly than arrays assembled using wt H4 and similarly to arrays containing N-terminally truncated H4. Acetylation of the N-terminal tail of H4 could thus be an essential step in opening the chromatin in HD50myl cells.

Finally, it is worth noting that in all the studied cells, no core histone acetylation appears at either the 5′ or 3′ MAR. This apparent lack of activity is consistent with the observation that they are not insulators with enhancer-blocking and/or barrier activities, a conclusion drawn from noting that their deletion from a wild-type construct containing all the *cis*-control elements had no effect on macrophage specificity or copy

Downloaded from http://nar.oxfordjournals.org/ at University of Birmingham on June 1, 2015

number dependence of lysozyme expression in transgenic mice (44).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

Both the Portsmouth and Leeds laboratories acknowledge the BBSRC of Great Britain for research funding and for a Studentship for K.B. P.L. and C.L. are both supported by the Wellcome Trust and E.M. by a Research Bursary from the University of Portsmouth. Funding to pay the Open Access publication charges for this article was provided by the University of Portsmouth.

Conflict of interest statement. None declared.

REFERENCES

1. Bonifer,C., Jagle,U. and Huber,M.C. (1997) The chicken lysozyme locus as a paradigm for the complex developmental regulation of eukaryotic gene loci. *J. Biol. Chem.*, **272**, 26075–26078.
2. Lefevre,P., Melnik,S., Wilson,N., Riggs,A.D. and Bonifer,C. (2003) Developmentally regulated recruitment of transcription factors and chromatin modification activities to chicken lysozyme *cis*-regulatory elements *in vivo*. *Mol. Cell. Biol.*, **23**, 4386–4400.
3. Bonifer,C., Vidal,M., Grosveld,F. and Sippel,A.E. (1990) Tissue specific and position independent expression of the complete gene domain for chicken lysozyme in transgenic mice. *EMBO J.*, **9**, 2843–2848.
4. Phi-Van,L. and Stratling,W.H. (1988) The matrix attachment regions of the chicken lysozyme gene co-map with the boundaries of the chromatin domain. *EMBO J.*, **7**, 655–664.
5. Jantzen,K., Fritton,H.P. and Igo-Kemenes,T. (1986) The DNase I sensitive domain of the chicken lysozyme gene spans 24 kb. *Nucleic Acids Res.*, **14**, 6085–6099.
6. Chong,S., Riggs,A.D. and Bonifer,C. (2002) The chicken lysozyme chromatin domain contains a second, widely expressed gene. *Nucleic Acids Res.*, **30**, 463–467.
7. Doyon,Y., Selleck,W., Lane,W.S., Tan,S. and Cote,J. (2004) Structural and functional conservation of the NuA4 histone acetyltransferase complex from yeast to humans. *Mol. Cell. Biol.*, **24**, 1884–1896.
8. Graf,T.A., McNagny,K., Brady,G. and Frampton,J. (1992) Chicken 'erythroid' cells transformed by the gag-myb-ets-encoding E26 leukemia virus are multipotent. *Cell*, **70**, 201–213.
9. Huber,M.C., Graf,T., Sippel,A.E. and Bonifer,C. (1995) Dynamic changes in the chromatin of the chicken lysozyme gene domain during differentiation of multipotent progenitors to macrophages. *DNA Cell Biol.*, **14**, 397–402.
10. Baniahmad,A., Steiner,C., Kohne,A.C. and Renkawitz,R. (1990) Modular structure of a chicken lysozyme silencer: involvement of an unusual thyroid hormone receptor binding site. *Cell*, **61**, 505–14.
11. Kontaraki,J., Chen,H.H., Riggs,A. and Bonifer,C. (2000) Chromatin fine structure profiles for a developmentally regulated gene: reorganization of the lysozyme locus before trans-activator binding and gene expression. *Genes Dev.*, **14**, 2106–2122.
12. Beug,H., von Kirchbach,A., Doderlein,G., Conscience,J.F. and Graf,T. (1979) Chicken hematopoietic cells transformed by seven strains of defective avian leukemia viruses display three distinct phenotypes of differentiation. *Cell*, **18**, 375–390.
13. Hebbes,T.R., Clayton,A.L., Thorne,A.W. and Crane-Robinson,C. (1994) Core histone hyperacetylation co-maps with generalized DNase I sensitivity in the chicken beta-globin chromosomal domain. *EMBO J.*, **13**, 1823–1830.
14. Litt,M.D., Simpson,M., Recillas-Targa,F., Prioleau,M.N. and Felsenfeld,G. (2001) Transitions in histone acetylation reveal boundaries of three separately regulated neighboring loci. *EMBO J.*, **20**, 2224–2235.
15. Bulger,M., Schubeler,D., Bender,M.A., Hamilton,J., Farrell,C.M., Hardison,R.C. and Groudine,M. (2003) A complex chromatin landscape revealed by patterns of nuclease sensitivity and histone modification within the mouse beta-globin locus. *Mol. Cell. Biol.*, **23**, 5234–5244.
16. Elefant,F., Su,Y., Liebhaber,S.A. and Cooke,N.E. (2000) Patterns of histone acetylation suggest dual pathways for gene activation by a bifunctional locus control region. *EMBO J.*, **19**, 6814–6822.
17. Bruce,K., Myers,F.A., Mantouvalou,E., Lefevre,P., Greaves,I., Bonifer,C., Tremethick,D.J., Thorne,A.W. and Crane-Robinson,C. (2005) The replacement histone H2A.Z in a hyperacetylated form is a feature of active genes in the chicken. *Nucleic Acids Res.*, **33**, 5633–5639.
18. Parekh,B.S. and Maniatis,T. (1999) Virus infection leads to localized hyperacetylation of histones H3 and H4 at the IFN-beta promoter. *Mol. Cell*, **3**, 125–129.
19. Myers,F.A., Evans,D.R., Clayton,A.L., Thorne,A.W. and Crane-Robinson,C. (2001) Targeted and extended acetylation of histones H4 and H3 at active and inactive genes in chicken embryo erythrocytes. *J. Biol. Chem.*, **276**, 20197–20205.
20. Myers,F.A., Chong,W., Evans,D.R., Thorne,A.W. and Crane-Robinson,C. (2003) Acetylation of histone H2B mirrors that of H4 and H3 at the chicken beta-globin locus but not at housekeeping genes. *J. Biol. Chem.*, **278**, 36315–36322.
21. Tagoh,H., Melnik,S., Lefevre,P., Chong,S., Riggs,A.D. and Bonifer,C. (2004) Dynamic reorganization of chromatin structure and selective DNA demethylation prior to stable enhancer complex formation during differentiation of primary hematopoietic cells *in vitro*. *Blood*, **103**, 2950–2955.
22. Lefevre,P., Lacroix,C., Tagoh,H., Hoogenkamp,M., Melnik,S., Ingram,R. and Bonifer,C. (2005) Differentiation-dependent alterations in histone methylation and chromatin architecture at the inducible chicken lysozyme gene. *J. Biol. Chem.*, **280**, 27552–27560.
23. Hebbes,T.R., Thorne,A.W., Clayton,A.L. and Crane-Robinson,C. (1992) Histone acetylation and globin gene switching. *Nucleic Acids Res.*, **20**, 1017–1022.
24. Crane-Robinson,C., Myers,F.A., Hebbes,T.R., Clayton,A.L. and Thorne,A.W. (1999) Chromatin immuno-precipitation assays in acetylation mapping of higher eukaryotes. *Methods Enzymol.*, **304**, 533–547.
25. Thorne,A.W., Myers,F.A. and Hebbes,T.R. (2004) Native chromatin immunoprecipitation. *Methods Mol. Biol.*, **287**, 21–44.
26. Roh,T.Y., Ngau,W.C., Cui,K., Landsman,D. and Zhao,K. (2004) High-resolution genome-wide mapping of histone modifications. *Nat. Biotechnol.*, **22**, 1013–1016.
27. Pokholok,D.K., Harbison,C.T., Levine,S., Cole,M., Hannett,N.M., Lee,T.I., Bell,G.W., Walker,K., Rolfe,P.A., Herbolsheimer,E. *et al.* (2005) Genome-wide map of nucleosome acetylation and methylation in yeast. *Cell*, **122**, 517–527.
28. Raisner,R.M., Hartley,P.D., Meneghini,M.D., Bao,M.Z., Liu,C.L., Schreiber,S.L., Rando,O.J. and Madhani,H.D. (2005) Histone variant H2A.Z marks the 5′ ends of both active and inactive genes in euchromatin. *Cell*, **123**, 233–248.
29. Millar,C.B., Xu,F., Zhang,K. and Grunstein,M. (2006) Acetylation of H2AZ Lys 14 is associated with genome-wide gene activity in yeast. *Genes Dev.*, **20**, 711–722.
30. Schubeler,D., MacAlpine,D.M., Scalzo,D., Wirbelauer,C., Kooperberg,C., van Leeuwen,F., Gottschling,D.E., O'Neill,L.P., Turner,B.M., Delrow,J. *et al.* (2004) The histone modification pattern of active genes revealed through genome-wide chromatin analysis of a higher eukaryote. *Genes Dev.*, **18**, 1263–1271.
31. Viens,A., Mechold,U., Brouillard,F., Gilbert,C., Leclerc,P. and Ogryzko,V. (2006) Analysis of human histone H2AZ deposition *in vivo* argues against its direct role in epigenetic templating mechanisms. *Mol. Cell. Biol.*, **26**, 5325–5335.
32. Lutz,M., Burke,L.J., LeFevre,P., Myers,F.A., Thorne,A.W., Crane-Robinson,C., Bonifer,C., Filippova,G.N., Lobanenkov,V. and Renkawitz,R. (2003) Thyroid hormone-regulated enhancer blocking: cooperation of CTCF and thyroid hormone receptor. *EMBO J.*, **22**, 1579–1587.
33. Musri,M.M., Corominola,H., Casamitjana,R., Gomis,R. and Parrizas,M. (2006) Histone H3 lysine 4 dimethylation signals the transcriptional competence of the adiponectin promoter in preadipocytes. *J. Biol. Chem.*, **281**, 17180–17188.

Downloaded from http://nar.oxfordjournals.org/ at University of Birmingham on June 1, 2015

34. Liang,G., Lin,J.C., Wei,V., Yoo,C., Cheng,J.C., Nguyen,C.T., Weisenberger,D.J., Egger,G., Takai,D., Gonzales,F.A. *et al*. (2004) Distinct localization of histone H3 acetylation and H3-K4 methylation to the transcription start sites in the human genome. *Proc. Natl Acad. Sci. USA*, **101**, 7357–7362.
35. Bernstein,B.E., Kamal,M., Lindblad-Toh,K., Bekiranov,S., Bailey,D.K., Huebert,D.J., McMahon,S., Karlsson,E.K., Kulbokas,E.J.,III, Gingeras,T.R. *et al*. (2005) Genomic maps and comparative analysis of histone modifications in human and mouse. *Cell*, **120**, 169–181.
36. Roh,T.Y., Cuddapah,S. and Zhao,K. (2005) Active chromatin domains are defined by acetylation islands revealed by genome-wide mapping. *Genes Dev*., **19**, 542–552.
37. Schubeler,D., Francastel,C., Cimbora,D.M., Reik,A., Martin,D.I. and Groudine,M. (2000) Nuclear localization and histone acetylation: a pathway for chromatin opening and transcriptional activation of the human beta-globin locus. *Genes Dev*., **14**, 940–950.
38. Li,Z., Luo,Z. and Scharff,M.D. (2004) Differential regulation of histone acetylation and generation of mutations in switch regions is associated with Ig class switching. *Proc. Natl Acad. Sci. USA*, **101**, 15428–15433.
39. Zhang,C.L., McKinsey,T.A. and Olson,E.N. (2002) Association of class II histone deacetylases with heterochromatin protein 1: potential role for histone methylation in control of muscle differentiation. *Mol. Cell. Biol*., **22**, 7302–7312.
40. Avni,O., Lee,D., Macian,F., Szabo,S.J., Glimcher,L.H. and Rao,A. (2002) T(H) cell differentiation is accompanied by dynamic changes in histone acetylation of cytokine genes. *Nature Immunol*., **3**, 643–651.
41. Vermeulen,M., Walter,W., Le Guezennec,X., Kim,J., Edayathumangalam,R.S., Lasonder,E., Luger,K., Roeder,R.G., Logie,C., Berger,S.L. *et al*. (2006) A feed-forward repression mechanism anchors the Sin3/Histone deacetylase and N-CoR/SMRT corepressors on chromatin. *Mol. Cell. Biol*., **26**, 5226–5236.
42. Dorigo,B., Schalch,T., Bystricky,K. and Richmond,T.J. (2003) Chromatin fiber folding: requirement for the histone H4 N-terminal tail. *J. Mol. Biol*., **327**, 85–96.
43. Shogren-Knaak,M., Ishii,H., Sun,J.M., Pazin,M.J., Davie,J.R. and Peterson,C.L. (2006) Histone H4-K16 acetylation controls chromatin structure and protein interactions. *Science*, **311**, 844–847.
44. Bonifer,C., Yannoutsos,N., Krüger,G., Grosveld,F. and Sippel,A. (1994) Dissection of the locus control function located on the chicken lysozyme gene domain. *Nucleic Acids Res*., **22**, 4201–4210.

Downloaded from http://nar.oxfordjournals.org/ at University of Birmingham on June 1, 2015