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

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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 DNase I 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 DNase I 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
enrichers 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 enrichers, 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 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 (3), 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 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% foetal 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

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**Figure 1.** (A) The cell lines used and their relationship. (B) Relative mRNA levels of Gas41 and Lysozyme in the cell lines used here.
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...
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 immunoprecipitates 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 the case. Observation of such ‘bulk’ co-habitation is not unexpected, however, in the light of ‘genome-wide’ mapping of the case. Observation of such ‘bulk’ co-habitation is not unexpected, however, in the light of ‘genome-wide’ mapping of the case. Observation of such ‘bulk’ co-

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′ 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 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 enhancer. In this region of the locus, five very closely spaced ampiclons 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 enhancer. 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...
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'.
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...
Figure 5. Distributions of hyperacetylated H2B at the lysozyme locus in the four cell types. 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 (HD50mym) 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 HD50mym 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
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 in contrast to previously studied examples for which core histone acetylation appears at either the 5' or 3' MAR. The present observations show that acetylation of H4 in 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 appears during the viral induction of the human IFNγ 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 IFNγ 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 TH1 cells, nor at the IL4 gene characteristic of TH2 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(H)1 cells and at the promoter, enhancer and two DHS of the IL4 gene in T(H)2 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(H)2 cells, it is unfortunately impossible to judge whether H3 acetylation was subject to a ‘temporal lag’ relative to H4 acetylation in T(H)2 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
number dependence of lysozyme expression in transgenic mice (44).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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