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Core histone hyperacetylation co-maps with generalized DNase I sensitivity in the chicken β-globin chromosomal domain

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The distribution of core histone acetylation across the chicken β-globin locus has been mapped in 15 day chicken embryo erythrocytes by immunoprecipitation of mononucleosomes with an antibody recognizing acetylated histones, followed by hybridization probing at several points in the locus. A continuum of acetylation was observed, covering both genes and intergenic regions. Using the same probes, the generalized sensitivity to DNase I was mapped by monitoring the disappearance of intact genomic restriction fragments from Southern transfers. Close correspondence between the 33 kb of sensitive chromatin and the extent of acetylation indicates that one role of the modification could be the generation and/or maintenance of the open conformation. The precision of acetylation mapping makes it a possible approach to the definition of chromosomal domain boundaries.

Key words: active chromatin/chicken β-globin/histone acetylation

Introduction

Core histone acetylation is a reversible post-translational modification which has been associated with transcriptionally active chromatin (Allfrey *et al.*, 1964; Hebbes *et al.*, 1988; reviewed by Csordas, 1990; Turner, 1991). This is only one of several biochemical changes which must occur at the chromatin level within a given cell type to generate a transcriptionally competent genetic locus (Gross and Garrard, 1987; Grunstein, 1990; Felsenfeld, 1992; Kornberg and Lorch, 1992). Immunoprecipitation experiments using antibodies capable of recognizing acetylated core histones have shown the modification to be present on both potentially and transcriptionally active chromatin, but not on permanently repressed chromatin (Hebbes *et al.*, 1992; Clayton *et al.*, 1993). Recently it has been confirmed that repressed chromatin does not carry acetylated histone H4 by the demonstration that the human female inactive X chromosome fails to stain with antibodies to acetylated H4, in contrast to all the other chromosomes (Jeppesen and Turner, 1993). Experiments showing that high levels of acetylation are not conditional upon active transcription, but relate rather to the transcriptional competence of the gene (Hebbes *et al.*, 1992; Clayton *et al.*, 1993), suggest that the modification plays an 'enabling' role in transcriptional activation. A lack of direct dependence on active transcription has also been shown by genetic experiments with *Saccharomyces cerevisiae* in which a promoter mutation resulting in failure to transcribe the α gene in the HML locus did not lead to loss of histone acetylation at this gene in *sir*$^-$ strains in which silencing of this locus had been abolished (Braunstein *et al.*, 1993). A specific role for histone H4 acetylation in transcriptional activation at promoter sequences has been indicated by *in vitro* reconstitution experiments showing that the presence of modified H4 facilitates the binding of the transcription factor TFIIIA to its target sequence without nucleosome displacement (Lee *et al.*, 1993). A role for histone acetylation on nucleosomes in the region of promoters is also implied by the observation that CpG island chromatin carries exceptionally high levels of the modification (Tazi and Bird, 1990).

The importance of acetylation in the control of transcription can be inferred from genetic experiments with *S.cerevisiae* in which deletion of the histone H4 N-terminal region or substitution of the specific modifiable lysine residues resulted in a decrease in the activation of the *GAL1* and *PHO5* promoters (Durrin *et al.*, 1991). For histone H3, in contrast, corresponding deletions or substitutions allowed enhanced activation of *GAL1* and other GAL4-regulated genes at lower galactose levels (Mann and Grunstein, 1992). The significance of modification at specific lysine residues has also been shown by the use of immunofluorescence to demonstrate a highly irregular pattern of chromosomal staining in *Drosophila* with antibodies to different isoforms of acetylated histone H4 (Turner *et al.*, 1992).

We have studied previously the chicken β-globin locus where a switch occurs between transcription of the embryonic β^e gene, which is active in the 5 day embryo, and the adult β gene, which is active in the 15 day embryo. Immunoprecipitation of chromatin fragments from both of these stages of development using an antibody to acetylated histone demonstrated enrichment of sequences from both the active and inactive globin genes at each stage (Hebbes *et al.*, 1992). Whilst this result demonstrated that the poised, transcriptionally active and previously active β-globin genes carry acetylated histone, it did not address the question of whether high levels of acetylation are found elsewhere in the locus. At the resolution of the light microscope, strong modulation of H4 acetylation along the chromosome has been indicated by immunofluorescence experiments (Jeppesen and Turner, 1993). This study sets out to define biochemically the distribution of the modification by asking whether high levels of acetylation are simply restricted to the genes, or found throughout the chicken β-globin locus extending up to and beyond the limits of the domain, as defined by generalized sensitivity to DNase I digestion.

Results

Generalized DNase I sensitivity

The preferential sensitivity of active genes to DNase I digestion is well established, in particular for the chicken β-globin locus (Weintraub and Groudine, 1976). The extent of nuclease-accessible chromatin has been determined as ≈20 kb for chicken lysozyme (Jantzen *et al.*, 1986; Strätling *et al.*, 1986), ≈100 kb for chicken ovalbumin (Lawson *et al.*, 1982) and ≈47 kb for human apolipoprotein B (Levy-Wilson and Fortier, 1989). In the case of the chicken lysozyme and human apolipoprotein B genes, the boundaries of the domain correspond to matrix attachment regions (MARs) (Strätling *et al.*, 1986; Phi-Van and Strätling, 1988). This preferential sensitivity always extends beyond the genes themselves and in the case of the chicken β-globins this region is reported to extend from 8–10 kb upstream of β^{ρ} and to at least 7 kb downstream of β^{ϵ} (Stalder *et al.*, 1980). Within this domain there are also 12 hypersensitive sites (Reitman and Felsenfeld, 1990), the most 5′ of which is a constitutive site (HS4) contained within the recently described insulator element (Chung *et al.*, 1993), while the remainder are erythroid-specific.

To determine whether hyperacetylation extends to the limits of the locus we have firstly re-investigated the extent of general DNase I sensitivity at the chicken β-globin locus. Erythrocyte nuclei from 15 day embryos were digested with increasing quantities of DNase I and extracted genomic DNA was then cut with various restriction enzymes. The DNA was quantified, electrophoresed, Southern blotted and hybridized simultaneously with combinations of the probes P1–P10 (see Figure 3 for their location). Relative sensitivities to DNase I were assessed by measuring loss of hybridization signal for each of the bands detected on the Southern blots. Figure 1 shows the results from probings of four such transfers, in each of which a probe (P1) for fragment D was included. This probe detects a 1.6 kb fragment located 14 kb upstream of the β^{ρ} gene, in a region of chromatin found previously to be beyond the 5′ boundary of DNase I sensitivity (Stalder *et al.*, 1980). This fragment is relatively resistant to DNase I as expected, persisting throughout the digest with only a 2- to 3-fold loss in hybridization signal intensity. In addition, this fragment showed a similar resistance to DNase I as a 3.2 kb ovalbumin fragment, a gene inactive in this tissue, detected on the same Southern blot (data plotted in Figure 3). The band intensities measured from the autoradiographs in Figure 1 were then plotted relative to those of fragment D taken from the same transfer.

Figure 1a and b shows data for fragments located at the 5′ end of the domain. Fragment C1, 2.7 kb long, extends 5′ from the constitutive hypersensitive site HS4 and abuts fragment D. Fragment C1 appears to be essentially resistant, but shows a slightly higher rate of cutting than fragment D, but considerably less than the 4.6 kb $\beta^{\rho 1}$ fragment that encompasses the embryonic β^{ρ} gene. The boundary of DNase I sensitivity is therefore located between fragments $\beta^{\rho 1}$ and C1, and the slight reduction in intensity of C1 relative to D suggests that C1 might just overlap the domain boundary. Indeed, C1 just extends into the constitutive hypersensitive site HS4 and the insulator element. The fragment $\beta^{\rho 1}$ includes the hypersensitive site HS1 which is reported as present in 15 day embryo erythrocytes (Reitman and Felsenfeld, 1990). The rapid loss of $\beta^{\rho 1}$ might thus be solely due to this. However, Figure 1b shows the rate of loss of fragments B and B′, located just 3′ of fragment C1. These two fragments are detected simultaneously in a *Bam*HI–*Hin*dIII digest due to polymorphism at the *Bam*HI site internal to B′. Both B and B′ show high sensitivity relative to fragment D and whilst for B′ this might be due to the fact that, like fragment C1, it just extends into the hypersensitive site HS4, the short fragment B is devoid of hypersensitive sites. Taken together the data in Figure 1a and b indicate that the boundary of sensitivity lies between fragments B and D, and the slight cutting detected for fragment C1 probably indicates that it lies in the immediate vicinity of the boundary.

Figure 1c and d shows an assessment of the DNase I sensitivity at the 3′ end of the domain in a region where there are no hypersensitive sites. Fragment 23 (3.2 kb) shows a sensitivity similar to that of $\beta^{\rho 1}$, whereas fragment 28 (3.1 kb) is almost as resistant as fragment D. Figure 1d includes data for fragment $\beta^{\rho 2}$ (7.8 kb) that is longer than $\beta^{\rho 1}$ in both 5′ and 3′ directions: $\beta^{\rho 2}$ is no more, and perhaps even less, sensitive than $\beta^{\rho 1}$. These data demonstrate that fragments 28 and 23 flank the 3′ boundary of the DNase I-sensitive domain. To check that fragment 28 is indeed outside the sensitive region, we examined fragment 32 (3.6 kb), located just 3′ of fragment 28, and found it to be as resistant as fragment D.

To investigate DNase I sensitivity further inside the domain we used a number of other probes and the results are included in Figure 3. Fragment A (4.2 kb), located between fragments B and $\beta^{\rho 2}$, contains hypersensitive sites HS2 and HS3 and shows the expected high sensitivity with a rapid initial drop in intensity as seems typical for fragments including hypersensitive sites. The same is true for the much shorter $\beta^{H/A}$ fragment. Fragment β^{A}, that includes most of the adult β coding sequences, shows high sensitivity but does not include a hypersensitive site. The sum of these data shows that the DNase I sensitivity extends from ~10 kb upstream of β^{ρ}, in agreement with the earlier finding of Stalder *et al.* (1980), to ~9 kb downstream of β^{ϵ}, covering ~33 kb of chromatin in all.

The above assessment of the susceptibility of different regions of the domain to DNase I represents a simplification in that it does not take account of the fact that if two fragments had the same susceptibility to DNase I, the larger fragment should be lost from the hybridization signal at a faster rate simply due to its greater length. The quantification of this effect (Wood and Felsenfeld, 1982) assumes a uniform sensitivity over the whole length of the fragment and therefore fragments containing hypersensitive sites would not be expected to fit the model. Indeed, fragments A and $\beta^{H/A}$ show a very sharp initial drop in intensity which we attribute to the presence of hypersensitive sites. The same may be true of B′. The model of Wood and Felsenfeld compares the loss of fragment intensity with the drop in size of bulk DNA (as a measure of overall accessibility to nuclease) and was applied to yield individual sensitivity values, S. Comparison of S values, relative to that of fragment D (S^{R}), shows values of S^{R} from 2 to slightly >4 for fragments inside the domain, and values slightly <1 for fragments outside the domain. The 3.2 kb fragment from the ovalbumin gene gave an S^{R} of ~0.8. Comparison of S^{R} values across the domain boundaries is critical for definition of those boundaries. For example, at the 3′ end, fragment 28 has

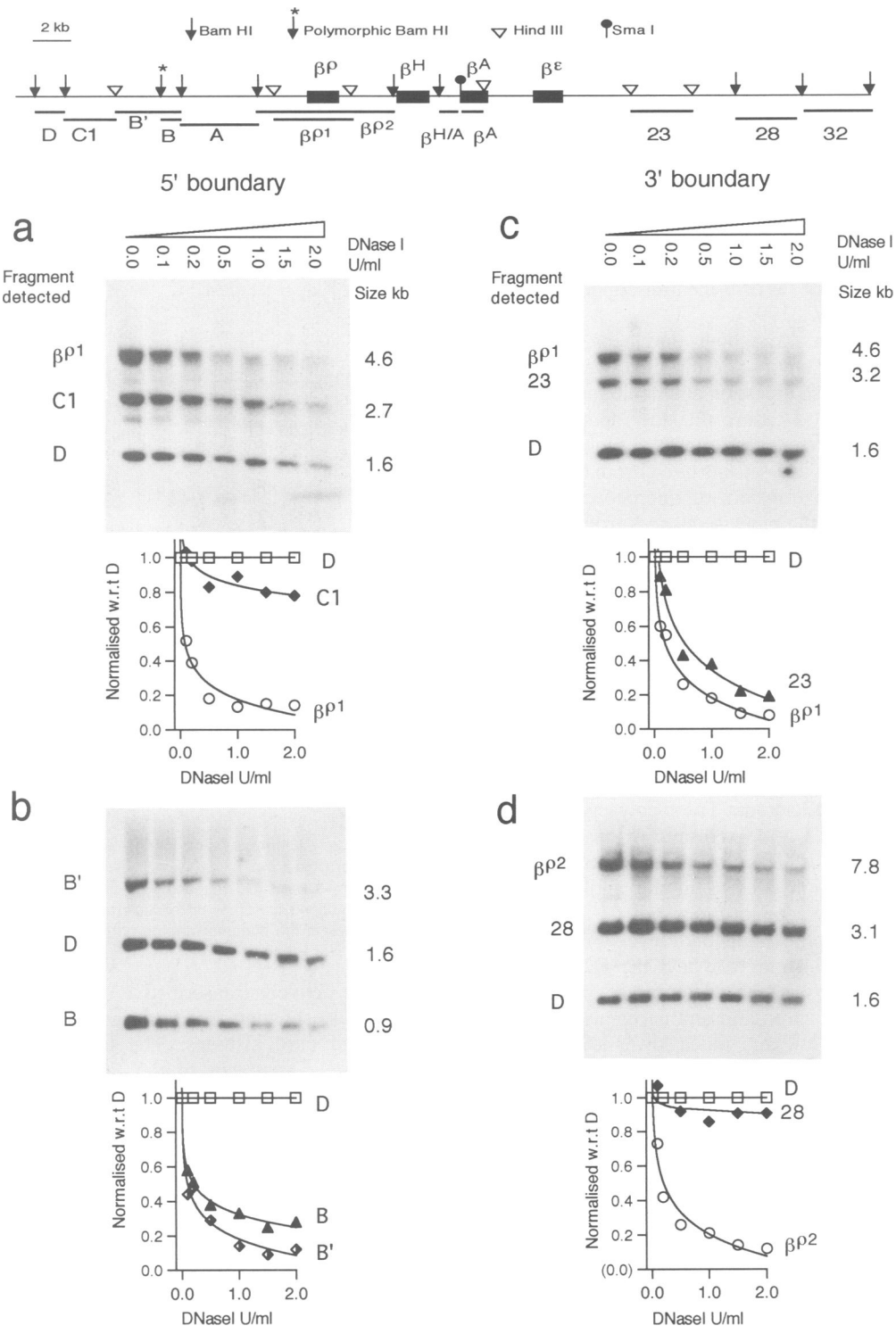

Fig. 1. Determination of DNase I sensitivity close to the 5′ and 3′ boundaries of the chicken β-globin locus. A combination of probes was used in each hybridization of Southern transfers of DNase I-digested and restricted DNA. Band intensities are plotted relative to that of fragment D. (**a**) Restriction with *Bam*HI+*Hin*dIII, probes P1, P2 and P5. (**b**) Restriction with *Bam*HI+*Hin*dIII, probes P1 and P3. (**c**) Restriction with *Bam*HI+*Hin*dIII, probes P1, P8 and P5. (**d**) Restriction with *Bam*HI, probes P1, P9 and P5.

S^R = 0.9, while fragment 23 has S^R = 2.0. At the 5′ end, fragment C1 has S^R = 0.8, while fragment B has S^R = 3.8. Fragments 28 and 23 are much the same size and the very considerable difference in sensitivity is plain from the uncorrected data. Fragment B is smaller than the control fragment D, but nevertheless disappears much more rapidly than D. None of the four fragments D, B, 23 or 28 contain hypersensitive sites and, even without correction for target size, the approximate location of the 5′ and 3′ boundaries of the DNase I-sensitive domain is clear.

Hyperacetylation mapping

We have studied previously the relationship of core histone acetylation to the developmentally regulated expression of the chicken β-globin genes. During embryogenesis globin expression switches from the embryonic to adult genes. This switch is not accompanied by a change in the high levels of core histone acetylation associated with these genes. In 5 day embryos, both the active β^{ϱ} and the poised β^{A} gene carry highly acetylated histones. By day 15, the expression has switched from embryonic to adult genes but the chromatin of the inactive β^{ϱ} gene still contains high levels of acetylated core histone, as does the transcriptionally active β^{A} gene (Hebbes *et al.*, 1992).

To map acetylation throughout the locus, chromatin fragments from 15 day embryo erythrocytes were prepared by micrococcal nuclease digestion and depletion of histone H1. It was important to ensure that the input chromatin contained a good representation of DNA sequences from both DNase I-sensitive and -insensitive chromatin regions. To this end, Southern transfers of micrococcal nuclease digests were probed with unique sequences from inside and outside the domain. This revealed a good representation of both classes of sequence in the mononucleosome population (data not shown). Mononucleosomes were purified on sucrose gradients and mixed with affinity-purified antibody to allow the formation of immunocomplexes. These were isolated by incubating with Protein A−Sepharose, collected by centrifugation and the unbound fraction in the supernatant was retained. After washing, the antibody-bound hyperacetylated chromatin was eluted with SDS and both DNA and protein extracted. The histones were analysed by electrophoresis in acetic acid/urea/Triton polyacrylamide gels (AUT PAGE) and the DNA fixed onto nylon membranes for hybridization analysis. The protein gel in Figure 2 shows the levels of acetylation present in the antibody-bound chromatin, a fraction which represents only 3% of the input chromatin. This fraction exhibits high levels of acetylation in the histones H3, H4 and H2B and the H2Az histone variant, demonstrating the high selectivity of the antibody. By contrast, the input chromatin and necessarily the 97% unbound chromatin show very much lower levels of core histone acetylation.

The DNA from input, unbound and antibody-bound fractions was quantified by UV spectrophotometry and equal masses of each fraction were taken for hybridization, as well as an equal mass of sonicated genomic DNA (designated 'Total'). Because the immunoprecipitations were performed using purified mononucleosomes, the resolution of the acetylation map is defined by the size of the probes used (always >200 bp long). Nevertheless, the resolution achieved is higher than for the DNase I sensitivity assay where the resolution is determined by the length of the fragment detected. Membranes were hybridized with the same probes used to detect fragments in the DNase I sensitivity experiments. The intensity of signal from the antibody-bound slot relative to that from the input slot (*B/I*) gives the enrichment generated by the antibody selection. Figure 3 summarizes the results of the acetylation mapping (in the lower half of the diagram) and also the DNase I sensitivity data. The probes and their location in the locus are shown above each membrane.

As expected from previous data (Hebbes *et al.*, 1992), strong enrichments, ≈20-fold, are observed when probing

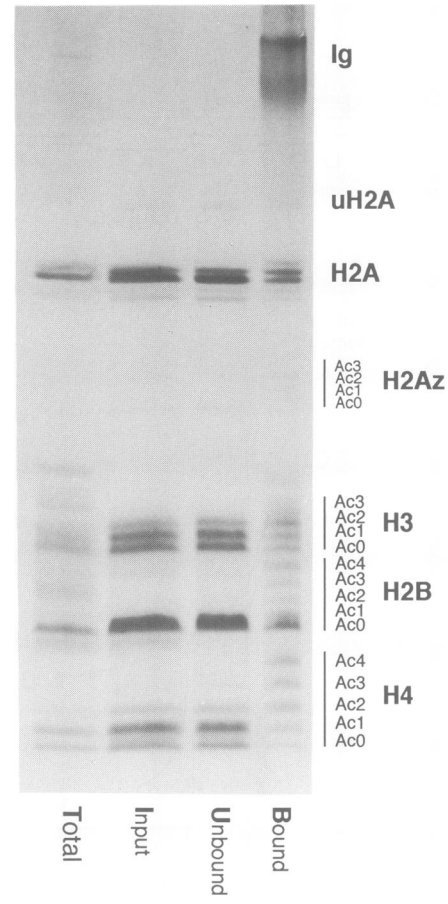

Fig. 2. Acetic acid/urea/Triton PAGE of proteins extracted from: total histone extracted from 15 day erythrocytes, input mononucleosomes purified on sucrose gradients and used for immunoprecipitation, unbound mononucleosomes in the supernatant and bound mononucleosomes in the immunocomplex.

with the actively transcribed β^{A} and the poised β^{ϱ} gene sequences, showing that these genes carry high levels of acetylated core histones. The intergenic sequence $\beta^{H/A}$ immediately upstream of the β^{A} gene also shows high enrichment. A strong hypersensitive site is contained within this region, a feature reflected in the rapid cutting in the DNase I sensitivity assay. Because the presence of a hypersensitive site indicates a region that is nucleosome-free, these data indicate that the nucleosomes surrounding the hypersensitive site contain highly acetylated core histones.

Probing for sequences beyond the gene cluster, both upstream and downstream, has revealed the presence of highly acetylated histone. Two probes, P3 and P4, located 7.1 and 3.4 kb upstream of β^{ϱ}, both show strong enrichments in the antibody-bound chromatin at levels similar to that of the active β^{A} gene (probe P7). Both of these probes are in DNase I-sensitive chromatin within fragments B and A, respectively. Beyond the 5′ boundary of DNase I sensitivity the probe P1, located 14 kb upstream of β^{ϱ} and within fragment D, is not preferentially selected by the antibody, showing the absence of hyperacetylation at that point. The probe P2, located 12 kb upstream of β^{ϱ} at the 5′ end of fragment C1, gives a similar result to P1.

At the 3′ end of the gene cluster the probe P8, located 4.7 kb downstream of β^{ϵ} and within the DNase I-sensitive

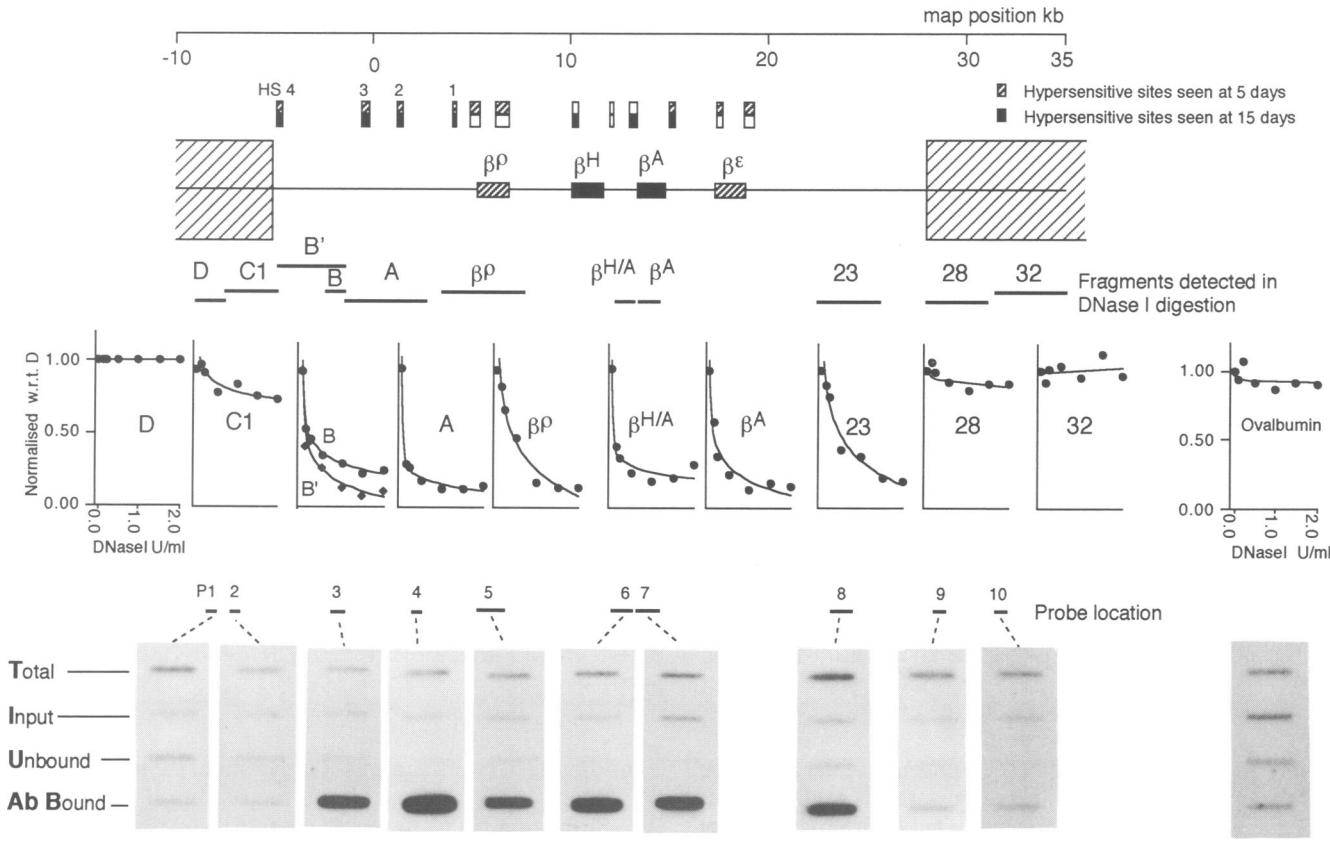

Fig. 3. The chicken β-globin locus showing the positions of the four genes and the hypersensitive sites (details from Reitman and Felsenfeld, 1990, and the map numbering system from Villeponteau *et al.*, 1982). The middle section of the figure shows the results of generalized DNase I sensitivity determinations with hybridization intensity plotted relative to that of fragment D. Data for ovalbumin were obtained using a *Bam*HI–*Hin*dIII digest and a cDNA probe. All data were obtained from a single set of DNase I digests. The lower band of the figure shows the results of acetylation mapping using probes P1–P10. 500 ng of DNA extracted from each of the fractions was applied to each slot: total sonicated genomic DNA from 15 day embryo erythrocytes, input DNA from mononucleosomes purified on sucrose gradients and used for immunoprecipitation, unbound DNA from mononucleosomes in the supernatant and bound DNA from hyperacetylated mononucleosomes in the immunocomplex.

fragment 23, shows high levels of enrichment, indicating that hyperacetylation extends downstream from the gene cluster. Beyond the boundary of DNase I sensitivity no hyperacetylation was detected with the two probes P9 and P10, located 9 and 13 kb downstream of β^{ϵ} within fragments 28 and 32, respectively, both of which are resistant to DNase I.

The DNase I assay shows that the 2.7 kb fragment C1 is essentially DNase I-resistant, although slightly less so than fragment D. The boundary of the domain may therefore be located just within fragment C1. We therefore probed the DNA from antibody-fractionated chromatin with sequences located immediately 5′ to the insulator element and thus very close to the constitutive hypersensitive site HS4. The results of this probing (probe P2a) are shown in Figure 4, together with the previous probings with P2 and P3. The results demonstrate clearly the presence of high acetylation levels at the P2a position, but not at P2. The boundary of hyperacetylation therefore lies in the 1 kb interval between these two probes and thus within 1.5 kb (about seven nucleosomes) of the 5′ end of the insulator element.

Discussion

The present data show that core histone hyperacetylation covers the whole of the DNase I-sensitive domain and is present not only on the genes of the chicken β-globin locus but is also on the long tracts of non-transcribed DNA between them. In the DNase I-resistant chromatin outside the domain the histones do not carry the high levels of acetylation seen within the DNase I-sensitive chromatin. Low levels of acetylation, e.g. mono and diacetyl H3 and H4 are very widespread in the genome and presumably present in much of the DNase I-insensitive chromatin. The presence of hyperacetylated core histones throughout the domain, covering precisely the region that is sensitive to DNase I digestion, implies that the modification is not exclusively concerned with transcription or the immediate preparation for it. This observation is consistent with the view that one of the roles the modification plays is to render a gene locus transcriptionally competent in a heritable fashion. It remains to be determined whether at all stages during cellular development a close correspondence is observed between DNase I sensitivity and hyperacetylation. Once a chromosomal domain has been rendered transcriptionally competent, a process that includes hyperacetylation of the core histones, the regulated expression of the individual genes can then be controlled by *cis* elements and their *trans*-acting factors.

That hyperacetylation co-maps with DNase I sensitivity over the whole of the domain suggests that the modification plays a role in defining the open chromatin conformation. This could be envisaged simply in one of two ways; either

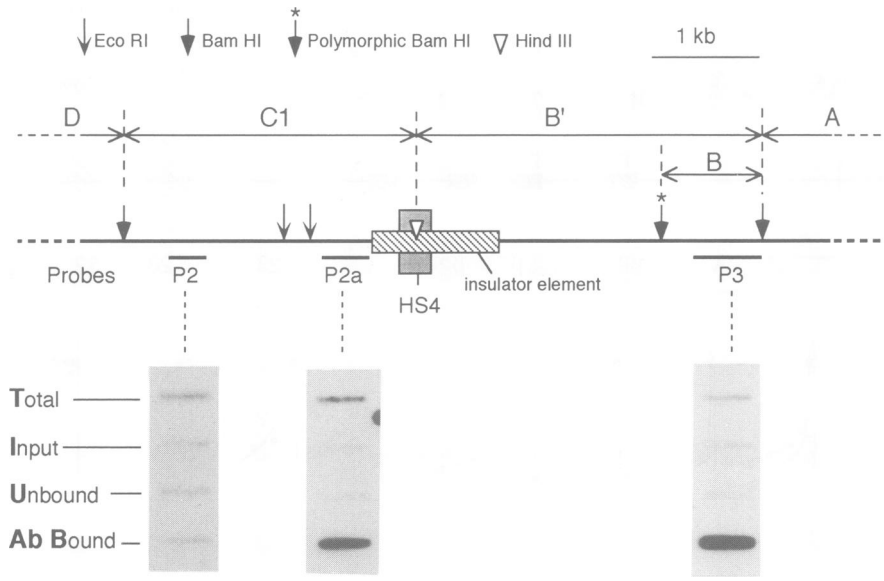

Fig. 4. Acetylation mapping close to the 5′ boundary of the chicken β-globin locus showing the positions of restriction fragments D, C1, B′, B and A with respect to the insulator element and the constitutive hypersensitive site HS4. The acetylation boundary maps to the region between probes P2 and P2a.

acetylation is the cause or is a consequence of the formation of the open structure. (i) Acetyltransferases, gaining access via the constitutive hypersensitive site (known to be present in inactive tissues e.g. brain; Reitman and Felsenfeld, 1990) where the domain is inaccessible to DNase I (Weintraub and Groudine, 1976; Wood and Felsenfeld, 1982), sequentially modify nucleosomes and in so doing open up the domain, perhaps because internucleosomal contacts involving core histone N-terminal tails are disrupted (Allan *et al.*, 1982). (ii) The closed conformation of the domain is opened by another mechanism (likewise initiated at the constitutive hypersensitive site), for example cooperative displacement of histone H1 (not necessarily its removal; Kamakaka and Thomas, 1990), and subsequently acetyltransferases gain access to the core histones. In either case the presence of the modification prevents reversion to the closed conformation and thereby 'enables' subsequent stages of transcriptional activation. Such a generalized role of core histone acetylation in the generation of domain accessibility would pave the way for modulation of acetylation at specific sites of individual histones so as to mark particular nucleosomes for removal or for interaction with *trans*-acting factors.

The DNase I accessibility experiments define the boundaries of the open conformation with somewhat greater precision than was revealed by earlier experiments (Stalder *et al.*, 1980). In particular, the 3′ boundary is defined to within ~2 kb at a distance of ~7.5 kb downstream of the end of the β^{ϵ} gene. The 5′ boundary is found to be close to the constitutive hypersensitive site HS4, 10 kb upstream of the start of the β^{ϱ} gene, which is within the interval determined by Stalder *et al.* (1980). Assessment of whether the limits of DNase I accessibility coincide precisely with the boundaries of core histone acetylation is limited by the resolution of the accessibility experiments, because the genomic fragments detected in the DNase I assay are longer than the probes used to monitor the acetylation of mononucleosomal fragments. Furthermore, the nature of the structures at the boundaries is uncertain. If the closed conformation is the 30 nm nucleosomal supercoil with six to seven nucleosomes per turn, then 1.2−1.4 kb of DNA could be at least partially exposed on the surface of the last superhelical turn. If the boundary region is matrix/scaffold attached (a MAR/SAR), the accessibility could be quite different. Such uncertainties make it difficult to give interpretations of the limits of accessibility. Mapping the limits of acetylation can be done, however, with a resolution given by the size of the probe or the length of the chromatin fragment selected by the antibody, whichever is the greater; such experiments can give specific biochemical information. The only limitations of the approach are that nucleosomes must be capable of being generated from the region of interest, which must itself consist of unique DNA sequences. The data in Figure 3 show that the 5′ limit of hyperacetylation corresponds fairly closely with the constitutive hypersensitive site HS4, and therefore with the insulator element defined by Chung *et al.* (1993). This element was shown to function solely as an insulator against adjacent chromosomal effects, i.e. as a border post, without having any intrinsic stimulatory or inhibitory properties, in the same way as shown for the specialized chromatin structure (scs) elements of *Drosophila* (Kellum and Schedl, 1991). The additional acetylation probing in Figure 4 shows that high levels of acetylation extend just beyond the insulator element. This raises the possibility that a full insulator includes more 5′ sequences than were tested by Chung *et al.* (1993), and this might explain why two copies of the insulator were needed at each end of the transgene to obtain a full insulation effect. Alternatively, as suggested by Chung *et al.* (1993), a 3′ boundary insulator may be necessary in addition to the 5′ insulator to achieve the effect of completely protecting the domain from external chromosomal influences. More detailed definition of the 3′ boundary of the domain by means of acetylation mapping could help resolve such questions.

Materials and methods

Antibodies

Affinity-purified polyclonal antibodies which recognize the epitope ε-acetyl lysine, and therefore all acetylated core histones, were prepared as described previously (Hebbes *et al.*, 1989).

Preparation of nuclei

Blood was collected from 15 day-old chicken embryos by vein puncture into PBS, 10 mM Na butyrate, 5 mM Na_3EDTA, 0.1 mM PMSF and 0.1 mM benzamidine. Cells were pelleted by centrifugation and, after washing in the above buffer omitting the Na_3EDTA, the erythrocyte cell pellet was resuspended in wash buffer (80 mM NaCl, 10 mM Tris−HCl pH 7.5, 10 mM Na butyrate, 6 mM $MgCl_2$, 0.1 mM PMSF and 0.1 mM benzamidine). Cells were lysed in 10 volumes of wash buffer supplemented with 0.1% Triton X-100. Nuclei were pelleted at 3000 r.p.m. for 5 min, resuspended in wash buffer and purified by centrifugation through a 30% sucrose cushion in the same buffer at 4000 r.p.m for 5 min at 4°C.

DNase I digestions

Nuclei were resuspended in digestion buffer (10 mM NaCl, 10 mM Tris−HCl pH 7.5, 10 mM Na butyrate, 3 mM $MgCl_2$) at a concentration of 0.5 mg/ml DNA, and digested with 0, 0.1, 0.2, 0.5, 1.0, 1.5 and 2.0 U/ml DNase I (Sigma) for 10 min at 37°C. Digestion was terminated with a final concentration of 5 mM Na_3EDTA and genomic DNA extracted using the method of Miller *et al.* (1988). For Southern analysis, the DNA was restricted with *Bam*HI alone, *Bam*HI and *Hin*dIII or *Bam*HI, *Hin*dIII and *Sma*I.

Preparation of mononucleosomes

Nuclei were resuspended in digestion buffer (10 mM NaCl, 10 mM Tris−HCl pH 7.5, 10 mM Na butyrate, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM PMSF and 0.1 mM benzamidine) at a concentration of 5 mg/ml DNA and digested with 200 U/ml micrococcal nuclease (Porton) for 10 min at 37°C. The digestion was terminated with 5 mM Na_3EDTA, the suspension chilled and immediately centrifuged at 13 000 *g* for 20 s, retaining the supernatant S1. The pellet was resuspended in lysis buffer (10 mM Tris−HCl pH 7.5, 10 mM Na butyrate, 0.25 mM Na_3EDTA, 0.1 mM PMSF and 0.1 mM benzamidine), incubated on ice for 2 min and recentrifuged as above. The supernatant S2 was combined with S1. The chromatin in these supernatants was depleted of H1 by incubating with 30 mg/ml Sephadex CM 25 cation exchange resin and the addition of NaCl to a final concentration of 50 mM for 1.5 h at 4°C under constant agitation. Following removal of resin−H1 complexes, mononucleosomes were purified by centrifuging the H1-depleted chromatin through 14 ml of 5−30% exponential sucrose gradients containing 10 mM Tris−HCl pH 7.5, 10 mM Na butyrate, 0.25 mM Na_3EDTA, 100 mM NaCl, 0.1 mM PMSF and 0.1 mM benzamidine at 40 000 r.p.m. (Beckman SW40) for 18 h at 4°C. This nucleosomal preparation procedure typically releases >80% of the nuclear DNA for the H1 depletion step and 25% of this is recovered from the sucrose gradients as the mononucleosomal fraction.

Immunoprecipitation of mononucleosomes

Immunoprecipitations were performed as described previously (Hebbes *et al.*, 1988, 1992). Mononucleosomes containing 400 μg DNA were incubated with 100 μg affinity-purified anti-acetylated H4 antibody in 750 μl incubation buffer (50 mM NaCl, 10 mM Tris−HCl pH 7.5, 10 mM Na butyrate, 1 mM Na_3EDTA, 0.1 mM PMSF and 0.1 mM benzamidine) for 2 h at 4°C under constant agitation. 50 mg washed Protein A−Sepharose (Sigma) were added to this and the suspension incubated for a further hour at 4°C under constant agitation. The immunocomplexes were collected by centrifugation (13 000 *g* for 2 min) and the unbound fraction in the supernatant retained. Resin pellets were washed five times with 1 ml incubation buffer. Chromatin and antibody bound to the Protein A−Sepharose beads were released by resuspending the pellet in 150 μl incubation buffer containing 1.5% SDS, followed by incubation at room temperature for 15 min. The Protein A−Sepharose beads were removed by centrifugation and the released material (the antibody-bound fraction) retained. The pelleted beads were washed with a further 150 μl incubation buffer containing 0.5% SDS, and the supernatant obtained after centrifugation (12 000 *g* for 2 min) was combined with the first. DNA from all chromatin fractions was obtained by two phenol/chloroform and one chloroform extraction, followed by ethanol precipitation. Proteins from all chromatin fractions were isolated from the phenol/chloroform phase of the initial phenol/chloroform extraction by the addition of 1/100th the volume of 10 M HCl followed by precipitation with 12 volumes of acetone. Precipitates were washed twice with acidified acetone (6:1, acetone:100 mM HCl) and finally three times with dry acetone before drying under vacuum.

Slot blots

DNA samples, quantified by UV spectrophotometry, were denatured in 0.5 M NaOH, 1.5 M NaCl for 10 min at 37°C and 1 min at 100°C. Samples were then applied to Biodyne B membranes (Pall) using a slot blot manifold (Bio-Rad), loading 0.5 μg DNA/slot. The filters were immersed in 0.5 M NaOH, 1.5 M NaCl for 5 min and then in 0.5 M Tris−HCl pH 7.2, 1.5 M NaCl, 10 mM Na_3EDTA for 30 s before blotting dry and baking at 80°C for 30 min.

Southern blots

Following restriction cleavage, the concentration of DNA digested by DNase I was determined using UV spectrophotometry and 4 μg DNA/track was electrophoresed through a 1% agarose gel. Following electrophoresis, the gel was incubated in 0.5 M NaOH, 1.5 M NaCl for 30 min and the DNA transferred to Biodyne B membranes in 20 × SSC. After transfer, the membranes were rinsed in 2 × SSC, blotted dry and baked at 80°C for 30 min.

Probes

Genomic probes from the chicken β-globin locus were obtained from several plasmids, as detailed below.

P1: 450 bp, *Nco*I−*Bam*HI, at −7.9 to −7.5, pCBG D, fragment detected D.
P2: 340 bp, *Sac*I, at −7.0 to −6.7, pCBG C, fragment detected C1.
P2a: 480 bp, *Sac*I, at −5.5 to −5.0, pCBG C.
P3: 600 bp, *Sac*I, at −2.0 to −1.4, pCBG B, fragments detected B and B′.
P4: 400 bp, *Bam*HI−*Eco*RI, at 2.1 to 2.5, pCBG A, fragment detected A.
P5: 1350 bp, *Hpa*II, at 5.4 to 6.7, pB2H9, fragments detected β^{e1} and β^{e2}.
P6: 1000 bp, *Eco*RI−*Sma*I, at 12.2 to 13.2, pB2H9, fragment detected $\beta^{H/A}$.
P7: 1100 bp, *Hin*dIII−*Sma*I, at 13.2 to 14.3, pBIEH1, fragment detected β^{A}.
P8: 1000 bp, *Kpn*I−*Pst*I, at 23.3 to 24.3, pCBG 23, fragment detected 23.
P9: 500 bp, *Bam*HI−*Eco*RI, at 28.0 to 28.5, pCBG 28, fragment detected 28.
P10: 500 bp, *Nco*I, at 31.5 to 32.0, pCBG 32, fragment detected 32.

An 850 bp cDNA fragment of ovalbumin from pOV 230 was used to probe for inactive gene sequences.

Hybridizations

Hybridizations were performed using 50 ng of probes labelled by random priming to specific activities of $4-8 \times 10^8$ d.p.m./μg at a concentration of 10 ng/ml. Filters were prehybridized for 60 min and hybridized for 2 h using Quick Hyb solution (Stratagene) at 68°C. Following hybridization, filters were washed as follows: twice in 2 × SSC, 0.1% SDS for 10 min at 42°C, once with 2 × SSC, 0.1% SDS for 30 min at 68°C, once with 0.2 × SSC, 0.1% SDS for 30 min at 68°C and finally twice with 2 × SSC, 0.1% SDS for 10 min at 42°C. All hybridization and washing procedures were performed in bottles in a hybridization oven (Hybaid). Filters were blotted dry and autoradiographed.

Electrophoresis

Proteins were analysed on 15% polyacrylamide acetic acid/urea/Triton (AUT) gels as described by Bonner *et al.* (1980) and stained with 0.1% Coomassie R 250 in 40% methanol, 10% acetic acid and destained in 7% acetic acid.

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