Islands of acetylated histone H4 in polytene chromosomes and their relationship to chromatin packaging and transcriptional activity

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Summary

The four histories of the nucleosome core particle are all subject to enzyme-catalysed, post-translational acetylation at defined lysine residues in their amino-terminal domains. Much circumstantial evidence suggests a role for this process in modifying chromatin structure and function, but detailed mechanisms have not been defined. To facilitate studies on the functional significance of histone acetylation, we have prepared antibodies specific for the acetylated isoforms of histone H4. Because of the extreme evolutionary conservation of H4, these antisera can be applied to a wide variety of organisms and experimental systems. In the present study we have used polytene chromosomes from the salivary glands of larvae of the midge Chironomus to examine the distribution of acetylated H4 in interphase chromatin. By indirect immunofluorescence, antisera to acetylated H4 labelled the four Chironomus chromosomes with reproducible patterns of sharply defined, fluorescent bands. An antiserum to non-acetylated H4 gave a completely different, more-diffuse labelling pattern. Thus, there are defined regions, or islands, in the interphase genome that are enriched in acetylated H4. Double-labelling experiments with two antisera specific for H4 molecules acetylated at different sites, showed that each antiserum gave the same banding pattern. Immunolabelling patterns were not dependent on the pattern of phase-dense bands characteristic of these chromosomes; strongly labelled regions could correspond to phase-dense bands (i.e. condensed chromatin), to interbands or, frequently, to band–interband junctions. Immunogold electron microscopy confirmed the immunofluorescence results and showed further that regions of relatively high labelling could be either transcriptionally active or quiescent, as judged by the presence or absence of ribonucleoprotein particles. Two rapidly transcribed genes on chromosome 4 of Chironomus form characteristic ‘puffs’, the Balbiani rings BRb and BRc. The antiserum to non-acetylated H4 gave diffuse labelling throughout these puffs, demonstrating the continued presence of this histone in these transcriptionally active regions. Antiserum to acetylated H4 strongly labelled the boundaries of BRb and BRc, and revealed clearly defined islands of increased H4 acetylation just within the expanded chromatin of the puffs. Labelling within the central region of each puff was much less intense. A similar pattern was observed in puffs on other chromosomes. Thus, increased H4 acetylation is not found throughout actively transcribed chromatin but occurs only at defined sites, possibly in the non-transcribed flanking regions. H4 acetylation is clearly not required for the passage of RNA polymerase through the nucleosome and we speculate that its role may be to facilitate the binding to DNA of polymerases and other proteins prior to the onset of transcription and possibly replication.

Key words: histone acetylation, transcription, polytene chromosomes, immunofluorescence.

Introduction

The histones of the nucleosome core particle are subject to a variety of enzyme-catalysed, post-translational modifications such as acetylation, phosphorylation and methylation (Wu et al. 1984). Eukaryotic cells invest a large amount of energy in these modifications (e.g. see Perry and Chalkley, 1982), and yet in no case have their effects on chromatin structure and function been clearly defined. Acetylation is one of the most dramatic and best studied of the post-translational changes (Loidl, 1988). Up to four acetate groups can be attached to specific lysine residues in the amino-terminal regions of the core histones H2B, H3 and H4 (or two in the case of H2A), causing a major
molecules in a typical mammalian cell carrying one or newly replicated chromatin (Sealy and Chalkley, 1979; al. 1983). Histone-DNA interactions within the core particle (Simp- ulations, but do show changes consistent with a loosening of general, been found to exhibit major structural alter- been hyperacetylated by butyrate inhibition have not, in general, been found to exhibit major structural alter- ations, but do show changes consistent with a loosening of histone-DNA interactions within the core particle (Simp- son, 1978; Muller et al. 1982; Imai et al. 1986; Ausio and van Holde, 1986), possibly with partial dissociation of the core DNA (Bode et al. 1983).

Increased levels of histone acetylation are found in newly replicated chromatin (Sealy and Chalkley, 1979; Cousens and Alberts, 1982; Annunziato and Seela, 1983; Allis et al. 1986) and acetylation of newly transcribed chromatin (Pederson et al. 1986; Ridsdale and Davies, 1987; Ip et al. 1988; Hebbes et al. 1988). However, mechanisms whereby histone acetylation may influence transcription, or chromatin assembly and remodelling, remain to be eluci- dated. Recent evidence suggests that the effects of histone acetylation may depend on which residues are acetylated as well as on the overall level of acetylation (Pesis and Matthews, 1986; Johnson et al. 1987; Turner, 1988).

To develop a new approach for investigation of histone acetylation, and particularly to facilitate studies on the functional significance of site-specific acetylation, we have prepared antisera that distinguish H4 molecules acety- lated at each of the four lysine residues used in vivo (Turner et al. 1989). By electrophoresis and Western blotting we have shown that the four H4 acetylation sites in mammalian cells are used in a preferred, though not exclusive, order (Turner et al. 1989), and that changes in both the level of acetylation and the frequency of use of particular sites occur as cells pass through metaphase (Turner and Fellows, 1989; Turner, 1989).

Antisera provide a unique opportunity to examine the distribution of acetylated H4 in chromatin by microscopi- cal techniques, an approach that may provide valuable clues to function. Labelling of metaphase chromosomes and interphase nuclei with antisera to acetylated H4 has suggested a non-uniform distribution of the antigen (Turner, 1988, and unpublished results). But the resol- ution obtainable with material from diploid cells does not permit examination of the relationship between acety- lation and local changes in chromatin packaging or the expression of specific genes. To do this we have turned to the giant polytene, interphase chromosomes found in salivary glands from the larvae of dipteran insects such as Drosophila and Chironomus (Hill et al. 1986).

Allis et al. (1986) found under phase contrast or after exposure to DNA stains exhibit banded patterns reflecting changes in DNA packaging and providing land- marks by which specific genes can be located. Rapid transcription at a particular gene locus is accompanied by expansion of the chromatin at that locus to form a 'puff', detectable at the light microscope level (Ashburner and Berendes, 1978). In the present paper we describe the distribution of acetylated H4 in polytene chromosomes from Chironomus using immunofluorescence and immu- noelectrophoresis. Our results show that acetylated H4 is not distributed uniformly throughout either the chromosome or the rapidly transcribed chromatin of puffs, but occurs instead in clearly defined regions of the inter- phase genome.

Materials and methods

Antisera

The preparation and characterisation of rabbit antisera to acety- lated histone H4 have been described in detail (Turner and Fellows, 1989; Turner et al. 1989).

Polytene chromosome preparation

Chironomus larvae were taken from local ponds and maintained in the laboratory at 18°C for up to two weeks. They were identified as C. thummi on the basis of chromosome banding (Hagele, 1975) and the presence of the nuclear organiser region on chromosome 4 (see Results). Salivary glands were removed from fourth instar larvae and placed in ice-cold Chironomus Ringer's solution, pH 6.3 (Robert, 1975), for up to 20 min. Chromo- somes were prepared as outlined below, the procedure being based on those described by Robert (1975), Silver and Elgin (1979) and Kurth et al. (1983).

Glands were transferred to the wells of a flexible, conical- bottomed, polyvinyl microtitre plate (Dynatec) and treated by sequential addition and removal of about 250 µl of the following solutions: (1) Chironomus Ringer's, pH 6.3, plus 2% Nonidet P-40, 1% Triton X-100 (10–20 min); (2) Chironomus Ringer's, pH 7.3, plus 2% Nonidet P-40, 1% Triton X-100, 3.2% formaldehyde (10–20 min); (3) 45% acetic acid, 10 mM MgCl2 (80 min).

Fixed glands were placed between a coverslip and a glass microscope slide and the chromosomes released by firm pressure with lateral movement of the coverslip if necessary. The squashes were frozen in liquid nitrogen, the coverslips prised off with a scalpel blade and the slides placed in 80% ethanol at 4°C. Chromosomes thus prepared could be stored for a few days before morphological deterioration became apparent.

Immunolabelling

Slides were immersed sequentially in 40% ethanol (10 min), Dulbecco's phosphate-buffered saline (PBS, 10 min) and blocking solution comprising PBS, 1% bovine serum albumin and 0.5% Triton X-100 (30–60 min). Chromosomes were covered with rabbit antisera to acetylated H4, appropriately diluted in blocking solution, and incubated in a moist atmosphere at 4°C for at least 4 h. Slides were thoroughly washed and the second antibody applied as above for 3 h. For most experiments we used fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Sigma) diluted x40 in blocking solution. Slides were washed and mounted in 50% glycerol in PBS, containing 2% DABCO (1,4- diazabicyclo[2,2,2]octane, Aldrich Chemical Co.) and 0.2 µg ml⁻¹ Hoechst 33342. But the H4 in salivary chromosome or the rapidly transcribed chromatin of puff, used for antibody production served as controls. After several
brief rinses in Tris-buffered saline containing 0.05 % Tween 20 (TBST) and 5 % normal goat serum (NGS), the coverslips were inverted over goat anti-rabbit IgG conjugated with 5 nm or 10 nm colloidal gold particles (BioCell Research Labs, Cardiff) and incubated overnight at 4 °C in a moist chamber. The final washes in TBST, 5 % NGS were followed by brief fixation in glutaraldehyde, post-fixation for 1 h in 1 % OsO₄, dehydration in ethanol and flat embedding in epoxy resin (Taab). When polymerised the resin block bearing the chromosomes could be peeled away from the Thermanox. Suitable spreads were identified by phase-contrast microscopy, trimmed and sectioned at 80–100 nm parallel to the block surface. Sections picked up on nickel grids were lightly stained in uranyl acetate and examined in a JEOL 100 CX-2 microscope.

Results

Antisera

The antisera originally designated R5 and R6 have been shown to be specific for the acetylated isoforms of H4 and to distinguish H4 molecules acetylated at particular sites (Turner and Fellows, 1989; Turner et al. 1989). Antiserum R6 is specific for H4 isoforms acetylated at lysine 5 whereas R5 contains two populations of antibodies, one against H4 molecules acetylated at lysine 12 and one against molecules acetylated at two or more sites (Turner and Fellows, 1989). To denote these specificities the antisera are now designated R6/5 and R5/12, respectively.

Antibodies to acetylated H4 label clearly defined chromosome regions

R5/12 and R6/5 labelled each of the four polytene chromosomes in Chironomus salivary gland nuclei with a clearly defined pattern of transverse bands. A typical example (in this case chromosome 1) is shown in Fig. 1. Two aspects of the labelling pattern are immediately apparent. First, the labelling intensity of different regions of the chromosome is extremely variable, with some regions (relatively small in number) showing particularly bright fluorescence, while others are essentially negative. Second, there is no simple correspondence between the immunolabelling pattern and the pattern of phase-dense bands. This relationship is examined in more detail below.

The pattern of fluorescent bands on a given chromosome was reproducible from one nucleus to another. This is

Fig. 1. Distribution of acetylated histone H4 in chromosome 1 of Chironomus revealed by immunolabelling with antiserum R6/5. A. FITC fluorescence (antibody distribution); B, corresponding phase-contrast image. The main part of each panel shows a complete chromosome 1. The inset shows a broken chromosome 1 from the same gland comprising regions A–D only (these regions are indicated in B). The arrows in A indicate equivalent regions (islands) of antibody-labelled chromatin on each chromosome. Bar, 10 μm.

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Fig. 2. Chironomus chromosomes immunolabelled with pre-immune rabbit serum. A. Hoechst 33342 fluorescence (DNA distribution); B, the same field photographed to show FITC fluorescence (antibody distribution). The print has been overexposed to reveal the very low level of fluorescence over both the chromosomes and background material. Bar, 10 μm.

Fig. 3. Part of a chromosome immunolabelled with antiserum R15/0 against non-acetylated H4. The three photographs are of the same chromosome and show: A, Hoechst 33342 fluorescence (DNA distribution); B, phase-contrast image; C, FITC fluorescence (antibody distribution). The arrows are identically positioned in each photograph. The major phase-dense band indicated by the arrows labels relatively weakly with the antibody (C). Bar, 5 μm.

Fig. 4. The effect on antibody labelling of omitting formaldehyde fixation. Salivary gland nuclei were incubated in 45% acetic acid for about 10 min before squashing and immunolabelling with antiserum R6/5 in the usual way. A. Hoechst 33342 fluorescence (DNA distribution); B, FITC fluorescence (antibody distribution). To illustrate the very weak labelling obtained with material not prefixed with formaldehyde, FITC fluorescence was photographed using an exposure appropriate for chromosomes labelled in the usual way. The negative has been printed so as to maximise contrast and show the distribution of the residual label. Bar, 10 μm.

Illustrated in Fig. 1, which shows both a complete chromosome 1, and in the inset, a fragment from a different nucleus that consists of regions A to D only. Equivalent major fluorescent regions are arrowed in each photograph. The correspondence between the labelling patterns of the two chromosomes is striking, particularly in view of their different degrees of compaction.

Pre-immune sera gave consistently weak labelling of polytene chromosomes and the associated extra-chromosomal material (Fig. 2). As a further test of the specificity of labelling, the synthetic peptide against which these antisera were raised was added at a concentration of up to 10 μg/ml prior to application of antisera to the chromosomes. This reduced binding to insignificant levels (not shown).

We have recently been able to prepare an antiserum against part of the N-terminal domain (residues 1–18) of non-acetylated H4 (Turner et al. 1989). This antiserum, designated R15/0, gave diffuse labelling of polytene chromosomes, with a tendency to label the phase-dense bands less intensely than the interbands. A representative chromosome region containing phase bands of varying density is shown in Fig. 3. The labelling pattern given by R15/0 was quite distinct from that obtained with antisera to acetylated H4. The pattern of sharply defined fluorescent bands characteristic of these antisera was never seen after labelling with R15/0.

Chromosome preparation procedures do not determine antibody labelling patterns

In order to prevent extraction of histones and to minimise their possible migration during chromosome preparation, most experiments were carried out with glands that had been lightly fixed in formaldehyde prior to acid treatment. Chromosomes that were not prefixed in this way, and which were then exposed to 45% acetic acid for 10 min or more, labelled very weakly with either R5/12 or R6/5, presumably due to extraction of H4 (Fig. 4). The complex relationship between residual antibody labelling and phase banding in these chromosomes was reminiscent of...
that seen in chromosomes prepared by the usual procedure. Neither changing the duration of acid treatment following formaldehyde fixation (from 10 to 20 min), nor substitution of acetic acid by butyric acid (45%) or trichloroacetic acid (20%), had any major effect on the characteristic labelling patterns generated by R5/12 and R6/5. Chromosomes prepared in 0.085 M HCl were unlabelled, suggesting that even after formaldehyde fixation H4 can still be extracted by strong acids. Omitting the acid-treatment step altogether resulted in chromosomes with poorly defined phase bands, but they still showed a characteristic pattern of bright and dark bands after immunolabelling with R5/12 (not shown).

Antisera R5/12 and R6/5 recognize the same chromosome domains

Because antisera R6/5 and R5/12 recognise H4 molecules acetylated at different sites (lysines 5 and 12, respectively) the chromosome banding pattern that they reveal may reflect either regions of generally increased H4 acetylation and/or use of different H4 acetylation sites in different chromosome domains. In the former case, the two sera should give the same or very similar labelling patterns on each of the four Chironomus chromosomes whereas in the latter the labelling patterns should be different.

In order to analyse directly the relative contributions of acetylation at lysines 5 and 12 to the overall pattern of H4 acetylation, we carried out a double-labelling experiment using the protocol outlined in Materials and methods. As shown in Fig. 5, the patterns of FITC fluorescence (R6/5 in the example shown) and TRITC fluorescence (R5/12) were virtually identical. The second antiserum applied, whether R5/12 or R6/5, always labelled less strongly, presumably because of steric hindrance caused by binding of the first antibody. (For this reason the double-labelling approach cannot give a quantitative comparison of the labelling intensities of the two sera at specific sites.) It is important to emphasise that in no instance, irrespective of which antiserum was applied first, did we observe strongly TRITC-labelled regions that were not also labelled with FITC. We conclude that, in general, levels of acetylation at lysines 5 and 12 vary in parallel.

The relationship between H4 acetylation and phase banding is complex

Analysis of a large number of chromosome preparations immunolabelled with R5/12 and R6/5 showed that islands of increased H4 acetylation could coincide with phase-dense bands, with interbands or with band–interband junctions. An informative section of a typical chromosome, labelled with R5/12, is shown in Fig. 6. To facilitate comparison of the phase-contrast and fluorescence images, equivalent positions on each image are marked (small arrows). Major phase bands are numbered 1–18.

The chromosome shown contains phase bands that are labelled (e.g. 1, 2, 7 and 18) or unlabelled (e.g. 10, 11 and 17). Unlabelled phase bands often had a prominent, antibody-labelled region closely juxtaposed to them, sometimes centred over the band–interband junction. For example, phase band 8 is sandwiched between two fluorescent bands, one of which (the left) clearly overlaps the band itself. Some fluorescent bands coincided with interbands (e.g. the interband between the closely adjacent bands 5 and 6) while others occurred in regions of the chromosome containing only minor phase bands (large arrow) or no clearly discernable phase bands.

Further comparison of immunolabelling and phase banding can be made by examining the chromosome shown in Fig. 10.

**Fig. 5.** Double labelling of chromosomes with antisera R6/5 and R5/12 against H4 acetylated at lysines 5 and 12, respectively. A. FITC fluorescence (distribution of antibody R6/5); B, TRITC fluorescence (distribution of antibody R5/12). Large arrows indicate examples of regions labelled strongly with both antibodies. The small chromosome (number 4) in the lower half of each panel contains two transcriptionally active regions, the Balbiani rings (BRb and BRc), and the nucleolar organiser region. The edge of the nucleolus (no) is just visible in B. The small arrows in A indicate fine, clearly defined islands of antibody-labelled chromatin within or closely adjacent to the transcriptionally active regions of each Balbiani ring. Bar, 10 μm.
Fig. 6. Part of a chromosome immunolabelled with antibody R6/12. The upper panel shows the fluorescence (FITC) image and the lower the corresponding phase-contrast image. Major phase bands are numbered 1–18. The arrows indicate the positions of selected fluorescent bands on each photograph. The larger arrow indicates a well-defined fluorescent band in a region of the chromosome containing only minor phase bands. Arrows were located by tracing the fluorescent image onto a sheet of thin, transparent plastic and marking the positions of bands with pinpricks. The tracing was then overlaid onto the phase-contrast image and the positions of the fluorescent bands were again marked. For most chromosomes the intensity of FITC fluorescence was such that the relationship between phase and fluorescent bands could be examined directly by viewing the phase and fluorescence images simultaneously.

Localisation of acetylated H4 at the electron-microscope level

The distribution of antibody between bands and interbands was examined in more detail by electron microscopy. Chromosomes were prepared and labelled as for immunofluorescence, except that gold-conjugated anti-rabbit IgG was used as the second antibody. In Fig. 7A antibody labelling (i.e. the density of gold particles) is particularly pronounced in the interband region (defined by arrows) to the left of the major central band. The interband to the right of this band is comparatively weakly labelled, as is the chromat on the extreme left of the figure. Gold particles are located close to, or over, material of moderate electron density but relatively rarely over the condensed chromat on of the bands. The interbands shown all contain characteristic granules of up to 50 nm in diameter (arrowheads), presumably RNP particles (Hill et al. 1986), and are therefore likely to be transcriptionally active. There was no obvious relationship between the location or frequency of RNP particles and gold particles in these sections. As shown in Fig. 7B, antisera to acetylated H4 also labelled regions of the chromosome in which RNP particles were absent and which were presumably transcriptionally quiescent.

H4 acetylation in rapidly transcribed chromatin

Rapid transcription of certain genes in the polytene chromosomes of *Chironomus* and other diptera is associated with dramatic changes in chromatin compaction. 'Puffs' of expanded chromatin, easily visible by light microscopy, form around these loci and permit the ready identification of regions of high transcriptional activity. Chromosome 4 of *C. thummi*, the smallest chromosome, contains two particularly prominent puffs, the Balbiani rings, designated BRb and BRc (Hagele, 1975). These code for proteins required for construction of the tubes in which the larvae live and account for most of the non-nucleolar RNA synthesised by the salivary gland (Serfling et al. 1983, and references therein). Chromosome 4 also contains the nucleolar organiser region.

An example of chromosome 4 labelled with R6/5 is shown in Fig. 8. The figure shows the two Balbiani rings (BRb and BRc) and part of the nucleolus and nucleolar organiser region (NOR). One telomeric region is doubled back underneath the nucleolus and its chromat is intermingled with the upper part of BRc. In our preparations BRc was invariably more expanded than BRb. The comparatively weak staining of BRc with Hoechst (Fig. 8A) is typical, as is the separation of the paired homologous chromosomes at the puff boundaries (see also Fig. 9).

Within the Balbiani rings, labelling with antisera R6/5 was most intense at, or adjacent to, the puff boundaries. The central regions of both BRb and BRc were comparatively weakly labelled (Fig. 8C). Similar results were obtained with antibody R5/12, with antisera to H4 acetylated at lysines 8 and 16 (Turner et al. 1989) and with chromosomes fixed in formaldehyde but not exposed to acetic acid. The latter result argues strongly that selective histone extraction is not responsible for the generally weak immunolabelling of BRb and BRc.
Labelling at the puff boundaries was often distributed as bright, transverse, fluorescent bands. In the example shown in Fig. 8 these fluorescent bands are particularly prominent in and around the constriction between BRb and BRc. What appear to be remnants of very fine fluorescent bands nearer to the central region of BRb are indicated in C (arrowhead). Distinct fluorescent bands were also present at the boundary of BRb adjacent to region A and the boundary of BRc close to the nucleolar organiser region. (These are not well defined in Fig. 8 but good examples are shown in the double-labelled preparations in Fig. 5). The transverse bands of increased antibody labelling did not correspond to regions of increased chromatin density, as detected by phase or Hoechst banding (compare A, B and C). In fact, the clearly defined fluorescent bands in BRb shown in Fig. 8 fall in a region of particularly low Hoechst fluorescence.

The nucleolar organiser region was strongly labelled by R6/5 and R5/12 in extensively squashed preparations where access of the antibody was not prevented by nucleolar material. The labelling shown in Fig. 8C is typical. The absence of labelling in those parts of the chromosome underneath the nucleolus in Fig. 8 is presumably due to reduced accessibility.

As shown in Fig. 9, a very different pattern of labelling along chromosome 4 was obtained with antiserum R15/0, against non-acetylated H4. Diffuse labelling was observed over the whole chromosome, with the expanded chromatin of BRb labelling as strongly as other regions and BRc rather less so. Note that labelling with R15/0 extends over the whole of BRb and BRc as defined by phase contrast. The nucleolar organiser region (which has separated from BRc and is not included in the figure) was labelled with comparable intensity to BRb.
Two examples of puffs on chromosomes other than 4 are shown in Fig. 10. That shown in A–C is only moderately expanded and two phase-dense bands, strongly labelled with Hoechst, can be distinguished in its central region (A and B, respectively). The central region of the puff is labelled only weakly with R5/12 (C) whereas the borders are labelled comparatively strongly. The puff shown in D–F appears more fully expanded than that in A–C. Overall, the intensity of labelling with R5/12 is low in comparison to that of adjacent regions, but with a characteristic speckled distribution, suggesting clustering of the remaining acetylated H4. Such speckled labelling was also seen in Balbiani rings (Fig. 8).

**Discussion**

The results presented show that polytene chromosomes labelled by indirect immunofluorescence with antisera to acetylated histone H4, display a characteristic and reproducible pattern of fluorescent banding, which does not simply reproduce the pattern of light and dark bands seen under phase-contrast. We propose that this fluorescent banding pattern is attributable to the existence of defined regions, or islands, of interphase chromatin that are relatively enriched in the acetylated forms of H4. In what follows we consider the arguments for and against this proposition and its implications.
Chromatin structure and antibody labelling

Major regional variations in chromatin compaction are a characteristic feature of polytene chromosomes, with estimates of the relative amounts of DNA in phase-dense bands (condensed chromatin) and interbands (decondensed chromatin) ranging from 3:1 to 20:1 (discussed by Bautz and Kabisch, 1983). Such differences in chromatin compaction will inevitably influence the intensity of immunolabelling through effects on both the amount of antigen per unit area (epitope density) and epitope accessibility to antibody. However, chromatin compaction clearly does not, in itself, determine the labelling pattern given by antisera R5/12 and R6/5. Regions of intense labelling were found within both the condensed chromatin of some phase-dense bands and the relatively decondensed chromatin of some interbands. There was no simple and consistent relationship between chromatin condensation and the intensity of immunolabelling.

Immunolabelling can be influenced by fixation procedures, either through selective antigen extraction or destruction of epitopes. However, selective extraction of acetylated H4 is not consistent with the observed insensitivity of the labelling patterns (as opposed to the absolute level of labelling) to either the time of exposure to acid or the chemical nature of the acid used. Indeed, in the case of the transcriptionally active Balbiani rings, whose decondensed chromatin could be particularly susceptible to extraction effects, the general pattern of labelling remained the same in chromosomes not exposed to acid at all. Selective epitope destruction is also an unlikely explanation for our findings. Lysines in which the side-chain amino groups are acetylated cannot react with formaldehyde, and as the epitopes recognised by R6/5 and R5/12 do not contain, or depend on, adjacent non-acetylated lysine residues (Turner et al. 1989) they will not be selectively altered by formaldehyde fixation.

The acetylated sites detected by antisera R5/12 and R6/5 occur predominantly on the more highly acetylated H4 isoforms in mammalian cells (Turner et al. 1989). Could these antisera therefore reveal the distribution of only a small, highly acetylated population of acetylated H4 molecules in polytene chromosomes? We regard this as unlikely: first, because of the close similarity of the labelling patterns given by R5/12 and R6/5, and second, because antisera to the acetylation sites at lysines 8 and 16 also gave a distinctive banded labelling pattern, resembling those given by R5/12 and R6/5, in both Chironomus and Drosophila chromosomes (R.Simpson and B.Turner, unpublished). However, we cannot exclude the interesting possibility that some differences in site usage occur throughout the chromosome.

Transcriptionally active chromatin

It is now generally accepted that nucleosomes of normal structure and distribution are not present, or are very infrequent, in genes rapidly transcribed by RNA polymerases I or II (pol I and pol II; Scheer and Zentgraf, 1982; Reeves, 1984; Pederson et al. 1986). The pol II-transcribed chromatin of Balbiani rings is typical in lacking a clear nucleosomal repeat after endonuclease digestion and containing only occasional nucleosome-like particles detectable by electron microscopy (Widmer et al. 1984). However, immunolabelling with an antisera to histone H3 has revealed the continued presence of this histone in both BRb and BRc (Kurth et al. 1983). Results from various experimental systems have demonstrated the continued presence of histones in other genes transcribed by pol II (Scheer et al. 1979; Solomon et al. 1988; Nacheva et al. 1989) and pol I (Colavito-Shepansky and Gorovsky, 1983). Thus, at least some histones are retained in rapidly transcribed genes, but in a form that is not readily detectable by electron microscopy or nuclease protection. Models involving the formation of half-nucleosomes have been proposed to account for some of the properties of actively transcribed chromatin (Prior et al. 1983).

Our immunolabelling results are consistent with the continued presence of H4 in chromatin transcribed by pol I and pol II. Antiserum R15/0 to non-acetylated H4, labelled the transcriptionally active nucleolar organiser region and the Balbiani rings BRb and BRc. In both cases antibody was distributed throughout the expanded, transcriptionally active chromatin.

There is much evidence to show that histones associated with actively transcribed chromatin are more highly acetylated than histones in bulk chromatin (Pederson et al. 1986; Ridsdale and Davie, 1987; Ip et al. 1988; Hebbs et al. 1988). However, the distribution of acetylated histones within, or adjacent to, transcribed genes has not previously been defined, nor is it clear whether acetylation is an essential part of the transcription mechanism. The immunolabelling approach described above has enabled us to define in detail the distribution of acetylated H4 within the actively transcribed Balbiani ring genes of Chironomus. The results show that, while relatively increased H4 acetylation can indeed be found in these genes, it is

Fig. 9. Transcriptionally active chromatin of the Balbiani rings BRb and BRc immunolabelled with antibody R15/0 against histone H4. The figure shows a fragment of chromosome 4 that has become detached from the nucleolus. A. Hoechst 33342 fluorescence (DNA distribution). The Balbiani rings BRb and BRc are indicated. B. Phase-contrast. C. FITC fluorescence (antibody distribution). Bar, 10 μm.
confined to specific regions adjacent to the boundaries of the decondensed (puffed), transcriptionally active chromatin. We have recently obtained very similar results with the heat shock puffs in Drosophila polytene chromosomes (R.Simpson and B.M.T., unpublished data). In view of the results with R15/0, this pattern of antibody labelling cannot be attributed to loss of histones from the transcribed region, or to their extraction during fixation (discussed above), or to blocking of antigenic sites by the transcriptional machinery, unless, that is, acetylated H4 is selectively blocked.

The results presented strongly suggest that increased levels of H4 acetylation are not required in vivo for the passage of pol II through the coding regions of the Balbiani ring genes and at least some other rapidly transcribed genes. This conclusion is consistent with in vitro experiments showing that histone hyperacetylation is not required for transcription. Both bacterial and mammalian pol II have been shown to transcribe, apparently unimpeded, through nucleosomes containing normally acetylated histones (Losa and Brown, 1987; Lorch et al. 1987).

The long, continuous coding sequences characteristic of Balbiani ring genes apparently extend through most of the puffed region of both BRb and BRc (Wobus et al. 1980;
Baumlein et al. (1982). One would therefore expect to find non-coding regions and associated control elements only at the puff boundaries. Our results therefore raise the interesting possibility that islands of enhanced H4 acetylation are located in the non-transcribed flanking regions of the active Balbiani ring genes. If this is so, then increased H4 acetylation may be part of a mechanism that facilitates efficient binding of the various components of the transcriptional apparatus to flanking DNA, either by inducing changes in higher-order structure, for which there is little evidence (Dimitrov et al. 1986), or by facilitating the displacement of DNA from the histone core by competing proteins. The latter possibility is suggested by the association between hyperacetylation of core histones and their displacement by protamines during sperm maturation (Christensen and Dixon, 1982).

Nacheva and coworkers (1989), on the basis of histone–DNA cross-linking within the Drosophila hsp70 gene, have suggested that the N-terminal tails of histones serve to maintain histone–DNA contact during passage of polymerase through the nucleosome. This function presumably depends on the high net positive charge of the N-terminal tails and would be compromised by a reduction in this charge due to lysine acetylation. However, if the increased levels of H4 acetylation associated with transcriptionally active genes are found predominantly or exclusively in the flanking regions, while H4 associated with transcribed chromatin is normally acetylated or even under-acetylated, then a high net positive charge in the N-terminal tail will be retained where it is required. In a more general context, we may speculate that the islands of acetylated chromatin distributed through the interphase genome define those regions that must remain accessible, or responsive, to transcription factors, polymerases or other components of the machinery of transcription or replication, but are not necessarily undergoing either of these processes.

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References


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Note added in proof
A recent paper (Tazi, J. and Bird, A. (1990). Alternative chromatin structure at CpG islands. Cell 60, 909–920) shows that histones H3 and H4 are highly acetylated in the chromatin of 'CpG islands', parts of the genome known to be associated with the 5' regions of transcribed genes in vertebrate cells.