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Antibodies to defined histone epitopes reveal variations in chromatin conformation and underacetylation of centric heterochromatin in human metaphase chromosomes

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Abstract. Unfixed metaphase chromosome preparations from human lymphocyte cultures were immunofluorescently labelled using antibodies to defined histone epitopes. Both mouse monoclonal antibody HBC-7, raised against the N-terminal region of H2B, and rabbit serum R5/12, which recognizes H4 acetylated at Lys-12, gave non-uniform labelling patterns, whereas control antibodies against total histone fractions H4 and H1 produced homogeneous fluorescence. HBC-7 bound approximately uniformly to the bulk of the chromosomes, but the major heterochromatic domains of chromosomes 1, 9, 15, 16 and the Y showed significantly brighter fluorescence. Serum R5/12 indicated an overall reduction in acetylation of H4 in metaphase chromosomes compared with interphase nuclei, although some specific chromosomal locations had considerably elevated acetylation levels. Acetylation levels in the major heterochromatic domains appeared extremely low. To investigate further the differences noted in heterochromatin labelling, metaphases from cultures grown in the presence of various agents known to induce undercondensation of the major heterochromatic domains were similarly immunolabelled. Decondensed heterochromatin no longer exhibited higher than normal immunofluorescence levels with HBC-7. The higher resolution afforded by "stretching" the centromeric heterochromatin of chromosomes 1, 9 and 16 confirmed the low level of H4 acetylation in these domains. We consider the implications of these observations in relation to chromatin conformation and activity.

Introduction

The nucleosome is now well established as the fundamental repeating unit of eukaryotic chromatin. The nucleosome core particle comprises two molecules of each of the four core histones, H2A, H2B, H3 and H4, formed as an octameric disk around which approx. 145 bp of DNA forms 1.75 turns of a left-handed 10 nm helix (Richmond et al. 1984). This structure appears to be invariant and occurs in all species and classes of chromatin so far studied (reviewed by Felsenfeld 1978).

The spacer regions between nucleosome core particles are more variable, but in most chromatin a further 20 bp of DNA and one molecule of histone H1 (H5 in avian erythrocytes), together with the nucleosome core particle, form a larger structure, which has been called the "chromatosome" (Simpson 1978). The chromatosome thus contains about two complete turns of the DNA helix, with H1 "sealing off" the entry and exit points of the DNA. The chromatosome, together with linker DNA, whose length depends on the source of the chromatin, typically 0–80 bp, make up the complete nucleosome repeat unit. Only in yeast has histone H1 not yet been unequivocally identified.

It has been shown that in the presence of cations and H1, nucleosome core particles can undergo a further degree of organization, forming a 30 nm solenoid of 6–7 nucleosomes per turn (Thoma et al. 1979). 30 nm chromatin fibres have been detected by X-ray diffraction in both interphase and mitotic chromosomes, although it seems unlikely that actively transcribing or replicating chromatin could exist with this structure.

In addition to their simple forms, histones may also exist with various modifications, including phosphorylation, ADP-ribosylation, acetylation ubiquitin conjugation and methylation (Matthews 1988). DNA is also modified by methylation. Important and, as yet, unresolved questions are: how do these modifications affect chromatin structure; and, are such modifications regulators of gene activity? To date, more is known about the relationship between DNA methylation and gene structure (reviewed by Bird 1986) than about the role of histone modifications, although in neither case is the effect on chromatin structure well understood. Acetylation of histones seems to correlate with transcriptional activity and chromatin sensitivity to nuclease digestion, but whether acetylation is a prerequisite of chromatin activation or results secondarily from increased accessi-

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bility to acetyltransferases is not known. Acetylation alone appears to have only very minor effects on nucleosome and 30 nm fibre conformations (Csordas 1990).

One approach to studying histone modification at the macromolecular level is by immunofluorescence. Probing chromosomes or nuclei with antibodies recognizing specific histone modifications should detect differential patterns of modification. Antibodies recognizing particular histone epitopes might also show up differences in chromatin conformation through a variation in accessibility. Antisera raised against whole histone fractions have been available for some time, but are of limited use, since they merely confirm the biochemical evidence that all four core histones are present in all chromatin so far studied.

In the present study we used two antibodies against specific histone epitopes: HBC-7 is a mouse monoclonal antibody that recognizes the N-terminal region of histone H2B (Whitfield et al. 1986); R5/12 is a rabbit antiserum that binds preferentially to histone H4 acetylated at Lys-12 (Turner and Fellows 1989). Using an indirect immunofluorescence method on unfixed human mitotic chromosomes, we demonstrated that these two antibodies bind non-uniformly along the chromosome arms, in contrast to antisera raised against whole histone fractions, which bind uniformly. We also compared the binding of these antibodies to centromeric heterochromatin, both in normal metaphase chromosomes, and in chromosomes in which the heterochromatic domains had been induced to decondense by three different procedures. We discuss the implications of the patterns of fluorescence observed in relation to chromatin structure.

Materials and methods

Lymphocyte cultures. 20 ml of defibrinated blood were diluted with 10 ml of RPMI 1640 medium and layered onto 7.5 ml of Ficoll-hypaque. Lymphocytes were separated by centrifugation at 2600 rpm for 20 min and then washed twice with RPMI medium. The lymphocytes were pelleted at 1000 rpm for 12 min at each washing and carefully resuspended at each wash in 20 ml RPMI. 5 ml lymphocyte cultures were then established in RPMI 1640 supplemented with 10% (v/v) foetal bovine serum, 0.03% (w/v) glutamine, and stimulated with phytohaemagglutinin (PHA). Cultures were normally grown at 37° C for 72 h, and metaphases accumulated by adding Colcemid to a final concentration of 0.1 μg/ml 1–2 h before harvesting.

In some samples the heterochromatic domains of chromosomes 1, 9 and 16 were induced to decondense by exposure during the final hours of culture to either DAPI (4′, 6-diamidino-2-phenylindole) or 5-azacytidine. In an attempt to increase the total length of the chromosomes, we also used a fluorodeoxyuridine (FUdR)/uridine metabolic block, followed by release with thymidine and 5-azacytidine. Because the use of Colcemid to arrest the mitotic cells also reduced the degree of undercondensation, in the latter case we omitted Colcemid treatment. The protocols were as follows:

(a) 5-Azacytidine treatment: cells were grown in the presence of 0.37 μm 5-azacytidine for the final 5 h of culture before addition of Colcemid (Schmid et al. 1983).

(b) DAPI treatment: DAPI was added to a concentration of 100 μg/ml for 16–18 h before metaphase arrest with Colcemid (Rocchi et al. 1979).

(c) FUdR/uridine block: 18 h prior to harvesting, FUdR and uridine were added to cultures to final concentrations of 0.1 μM and 1 μM respectively. 5 h before harvesting, the block was removed by the addition of thymidine to 10 μM, at which time 5-azacytidine was also added to 0.37 μM. Colcemid was not added to these cultures.

Antibodies. The antibodies used were: HBC-7 (subclone B11-1B8), a mouse monoclonal antibody, class IgM, against human histone H2B (Turner 1982; Whitfield et al. 1986), in a hybridoma culture medium supernatant; R5/12, a rabbit antiserum against histone H4 acetylated at Lys-12 (Turner and Fellows 1989); R15/0, a rabbit antiserum against non-acetylated histone H4 (Turner et al. 1989); Ra120d, a rabbit antiserum against calf histone H1, prepared according to Stollar and Ward (1970). Ra120d was a gift from Dr. David Stollar.

Metaphase preparation and immunofluorescence. Cells were collected by centrifugation at 1000 rpm, and resuspended gently in 75 mM KCl hypotonic solution at a cell density of $1–2 \times 10^6$/ml. The cells were then incubated for 10–30 min at room temperature, after which they were kept on ice, and used promptly. A modification of the method of Perry and Thomson (1986) was used for the preparation of slides. Hypotonically swollen cells were diluted to approx. 2×10^5/ml, and 0.5 ml portions were centrifuged onto microscope slides for 10 min at 2000 rpm, using an Ames Cyto-Tek cytocentrifuge (Bayer Diagnostics UK) and 1 ml disposable sample chambers, incorporating absorbant cards with 6 mm square apertures. Immediately after centrifugation, slides were transferred to Coplin jars and immersed in 120 mM KCl, 20 mM NaCl, 10 mM Tris-HCl, pH 8, 0.5 mM EDTA, 0.1% (v/v) Triton X-100 (KCM; Gooderham and Jeppesen 1983) for a minimum of 10 min at room temperature, before proceeding.

HBC-7 culture supernatant was diluted 1/20 in KCM containing 10% (v/v) normal goat serum (NGS), and slides were incubated with 40 μl of the dilution for 1 h at room temperature in a humid chamber. For rabbit sera the dilution used was 1/100. After the first antibody reaction, the slides were gently washed three times (1 min, 5 min, 5 min) with KCM, then incubated with second antibody – either fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM for detection of HBC-7 binding, or FITC-goat anti-rabbit IgG for R5/12, R15/0 and Ra120d (both obtained from Sigma), diluted 1/20 in KCM + 10% NGS. The second antibody incubation was for 30 min, with other conditions as for the first antibody reactions. The slides were then washed three times with KCM as before, then fixed for 10 min in KCM containing 10% (v/v) formalin [approx. 4% (w/v) final concentration of formaldehyde], after which the slides were thoroughly rinsed with distilled water and air-dried.

Immunolabelled samples were counterstained with the DNA fluorochrome Hoechst 33258 as described previously (Jeppesen et al. 1978), then mounted for fluorescence microscopy in PBS AF3 (Citifluor), 50% (v/v) glycerol (Merck, for fluorescence microscopy). Photomicrographs were recorded on Kodak Tmax 400 using a Leitz Ortholux microscope fitted with UV epifluorescence optics, an HBO 50 W mercury vapour lamp and filter sets for UV and FITC fluorescence.

After photomicrography to record FITC fluorescence, coverglasses and mounting medium were removed, slides washed in distilled water, and chromosomes containing heterochromatic domains identified by methyl green/DAPI fluorescence, following the procedure of Donlon and Magenis (1983).

Results

Metaphase chromosome spreading procedure

Conventional methods of obtaining well spread preparations of metaphase cells for cytogenetic analysis are not, in general, suitable for immunofluorescence studies. A major difficulty is the reduction, or even total loss of

antigenicity likely to be caused by the rather harsh fixation conditions usually employed, for example, 3:1 methanol:acetic acid. There is also the possibility that, even if some antigenicity is retained, extraction of basic protein antigens by acidic fixatives, might occur. This could clearly lead to artefacts in the present study on histones.

We have developed the procedure described in this paper to avoid these potential problems. Although milder fixation methods have been described for immunofluorescence studies (e.g. Moroi et al. 1980), we have avoided fixation altogether for the antibody reactions by carrying them out in KCM ('potassium chromosome medium'; Gooderham and Jeppesen 1983). Metaphase chromosomes remain stable and retain their morphology in this buffer for several hours at room temperature. Fortuitously, KCM also provides an excellent antibody reaction environment (Jeppesen and Nicol 1986). A number of different techniques have been tried to obtain the initial metaphase chromosome spreads, but the one described here was found reproducibly to give the best results.

Metaphase arrested cells are equilibrated with hypotonic (75 mM) KCl solution and portions are centrifuged onto glass microscope slides through an aperture in a porous card, which absorbs excess liquid. A proportion of cells in the final preparations always appears distorted, with chromatin clearly decondensed, which we attribute to rupturing of cells at this stage and the consequent exposure of the cell contents to an increasing ionic strength during drying. The proportion of disrupted cells increases with decreasing number of cells on the slide, and in practice we have found that a cell density approaching a monolayer appears to minimize drying effects. It seems that close contact between cells provides them with mutual mechanical support.

After centrifugation, the slides are removed promptly, to avoid excessive drying, and pre-equilibrated in KCM before carrying out the immunolabelling reactions. KCM, which contains 0.1% Triton X-100, solubilizes the cell membrane, allowing unimpeded access to antibodies. Finally, after antibody reactions are completed and excess antibody has been removed, preparations are fixed with formaldehyde before counterstaining with a DNA fluorochrome and mounting for fluorescence microscopy.

The appearance of nuclei and chromosome spreads prepared in this way is markedly different from those obtained by conventional fixation. The most obvious difference is the small size of chromosomes, approximately one-half the size of conventional 3:1 methanol:-acetic acid fixed preparations. Also, chromosomes are not spread out over such a large area, and overlapping of chromosomes is frequent. Attempts to improve spreading by swelling in a more hypotonic medium resulted in more severe drying effects like those noted above. Alternative final fixation procedures (e.g. 100% methanol or methanol:acetic), in producing larger chromosomes, exacerbated the problem of overlapping. The small size of formaldehyde-fixed chromosomes is an advantage in this respect.

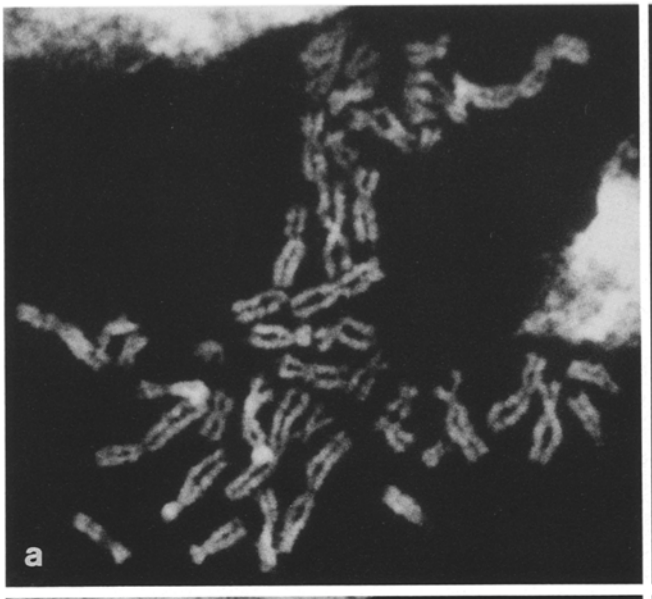
Decondensation of heterochromatic domains

We have used three different procedures to achieve undercondensation of the centromeric heterochromatic domains in chromosomes 1, 9 and 16: cells were cultured in the presence of 5-azacytidine, with or without a prior FUdR/uridine block, or the DNA-binding drug DAPI was added to the cell culture medium. Each procedure led to a proportion of metaphases containing one or more of these chromosomes exhibiting undercondensed heterochromatin, ranging from a slightly "stretched" appearance to highly extended heterochromatic domains, with the chromosome arms on either side widely separated. Examples of each of the procedures are illustrated in the figures. There was little significant difference in the appearance of undercondensed chromosomes prepared following treatments with DAPI or 5-azacytidine alone. The overall length of chromosomes was greatest following the FUdR/uridine block procedure, and some chromosomes prepared in this way exhibited undercondensation at multiple sites, probably corresponding to G-bands, throughout the genome (not shown here). Undercondensation of the heterochromatic domains of the acrocentric chromosomes 15 was also obtained, but undercondensation of Y chromosome heterochromatin of was not observed, although it has been described by others (Schmid et al. 1983).

Mouse monoclonal antibody HBC-7

Figure 1b shows the binding pattern of human metaphase chromosomes with mouse monoclonal antibody HBC-7, which specifically recognizes the N-terminal region of histone H2B (Whitfield et al. 1986), detected by indirect immunofluorescence. Most of the chromosomes seem more or less uniformly labelled, but the heterochro-

Fig. 1a–e. Binding of anti-histone H2B mouse monoclonal antibody HBC-7 to human metaphase chromosomes. Human lymphocytes were cultured, metaphases were prepared and antibody reactions were carried out as described in Materials and methods. **a** Metaphase from a normal lymphocyte culture stained with the DNA fluorochrome Hoechst 33258 and photographed by UV fluorescence. **b** Binding of HBC-7 to the same metaphase detected by indirect fluorescein isothiocyanate (FITC) immunofluorescence. The brightly fluorescing heterochromatic domains are *arrowed*. Small portions of adjacent interphase nuclei, at *top left* and *right*, show approximately the same intensity of immunofluorescence as the metaphase chromosome euchromatin. **c** UV fluorescence of metaphase depicted in **a** and **b** following treatment with methyl green/DAPI (4',6-diamidino-2-phenylindole). Chromosomes containing the major heterochromatic domains revealed by this procedure are identified. **d** Metaphase from a culture grown in the presence of DAPI to induce undercondensation of the heterochromatic domains (Hoechst 33258, UV fluorescence). Three "stretched" centromeres are *arrowed* and the chromosomes identified. **e** HBC-7 binding to the same metaphase as in **d**, detected by FITC indirect immunofluorescence. The *solid arrows* point to undercondensed heterochromatic domains that have lost their bright fluorescence. The *unfilled arrow* indicates a chromosome 9 heterochromatic domain that has not undergone decondensation and that retains its bright fluorescence. Bar represents 5 μm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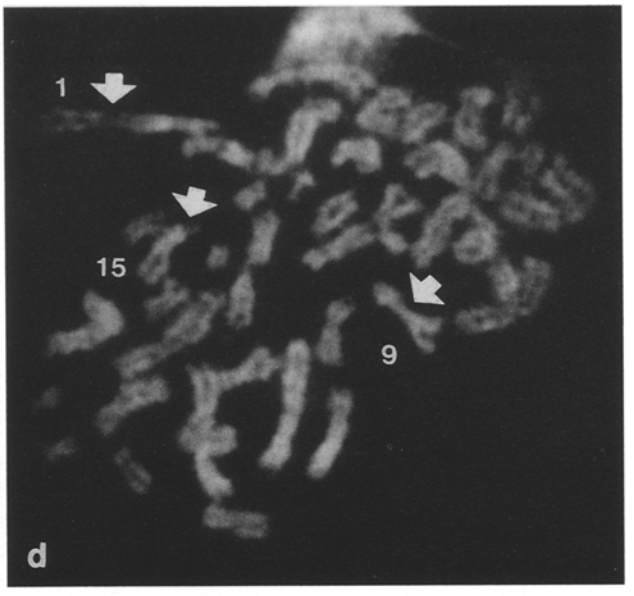
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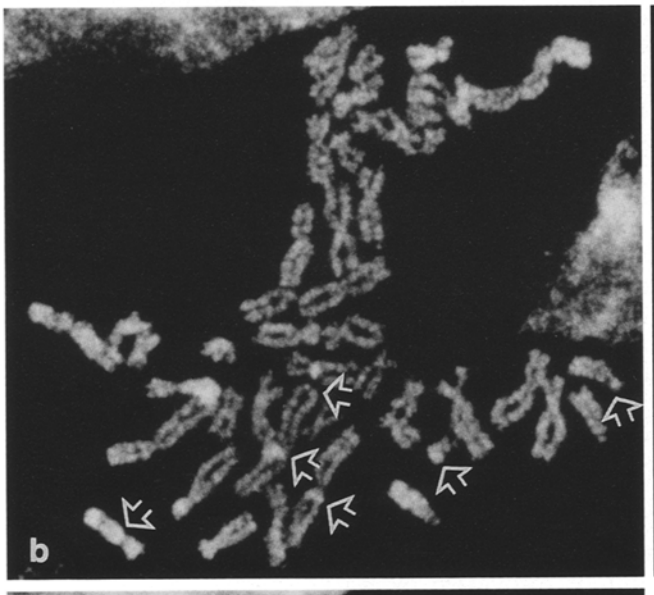
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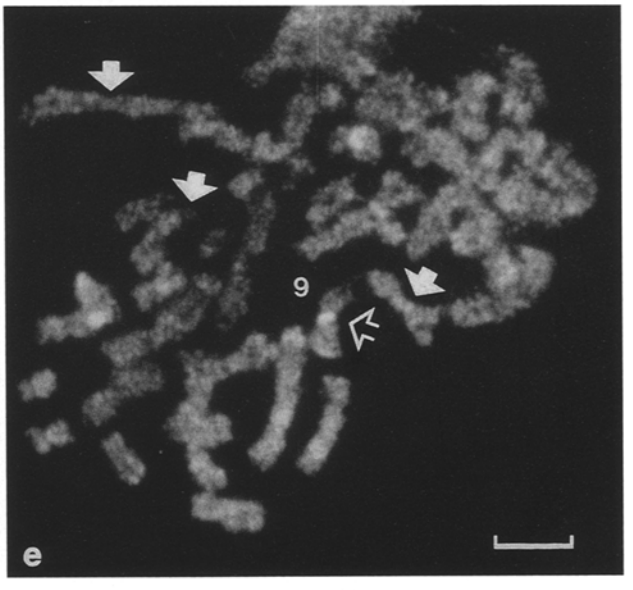
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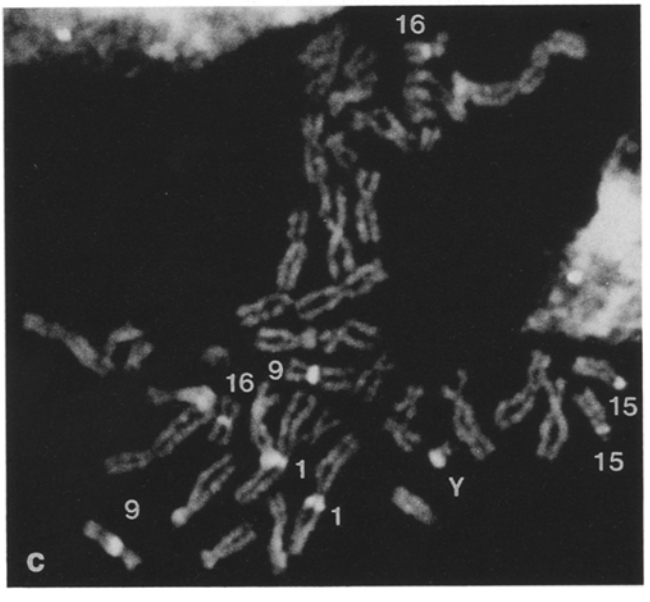
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matic domains of chromosomes 1, 9, 15, 16 and Y appear more intensely fluorescent. Portions of adjacent nuclei are also included in Fig. 1b, showing that the level of euchromatin immunofluorescence in mitotic chromosomes is approximately the same as that seen in interphase nuclei. In Fig. 1c, the same spread is shown stained by the methyl green/DAPI technique, which causes the major heterochromatic domains to fluoresce more strongly, showing the concordance with HBC-7 immunofluorescence. (In this particular photomicrograph, immunofluorescence of chromosome 16 heterochromatin with HBC-7 is not so pronounced, although in other examples, not shown here, the heterochromatin immunofluorescence of this chromosome is noticeably stronger.)

In contrast, when the same heterochromatic domains are undercondensed, as a result of use of any of the procedures described in Materials and methods, their level of immunofluorescence is reduced to that of the bulk of the euchromatin. This is illustrated in Fig. 1e, where a culture grown in the presence of DAPI was used for the preparation of metaphases. Two chromosomes 9 (shown in Fig. 1d, e) lie close together: one appears normally condensed, with intense immunofluorescence of the centromeric heterochromatin, whereas the other has undergone decondensation of the centromeric heterochromatin (as indicated by its stretched appearance), which has simultaneously lost its enhancement of HBC-7 binding.

The enhancement of fluorescence seen in normal chromosomes is not simply explained by a higher concentration of histone H2B in heterochromatin compared with euchromatin. Immunofluorescence using anti-histone H4 antibody (rabbit serum R15/0, see below), is not increased in heterochromatin, thus H4 is no more abundant in the heterochromatic domains than in euchromatin. Since the core histone composition of nucleosomes in heterochromatin from various species so far studied is identical to euchromatin (e.g. Zhang and Horz 1982; Omori et al. 1980; Musich et al. 1977), it is highly unlikely that human heterochromatin has an excess of H2B over H4. Thus the higher fluorescence observed with HBC-7 must reflect either a conformation or modification difference in histone H2B that is specific for these heterochromatic domains, and that allows enhanced binding of the antibody to its epitope.

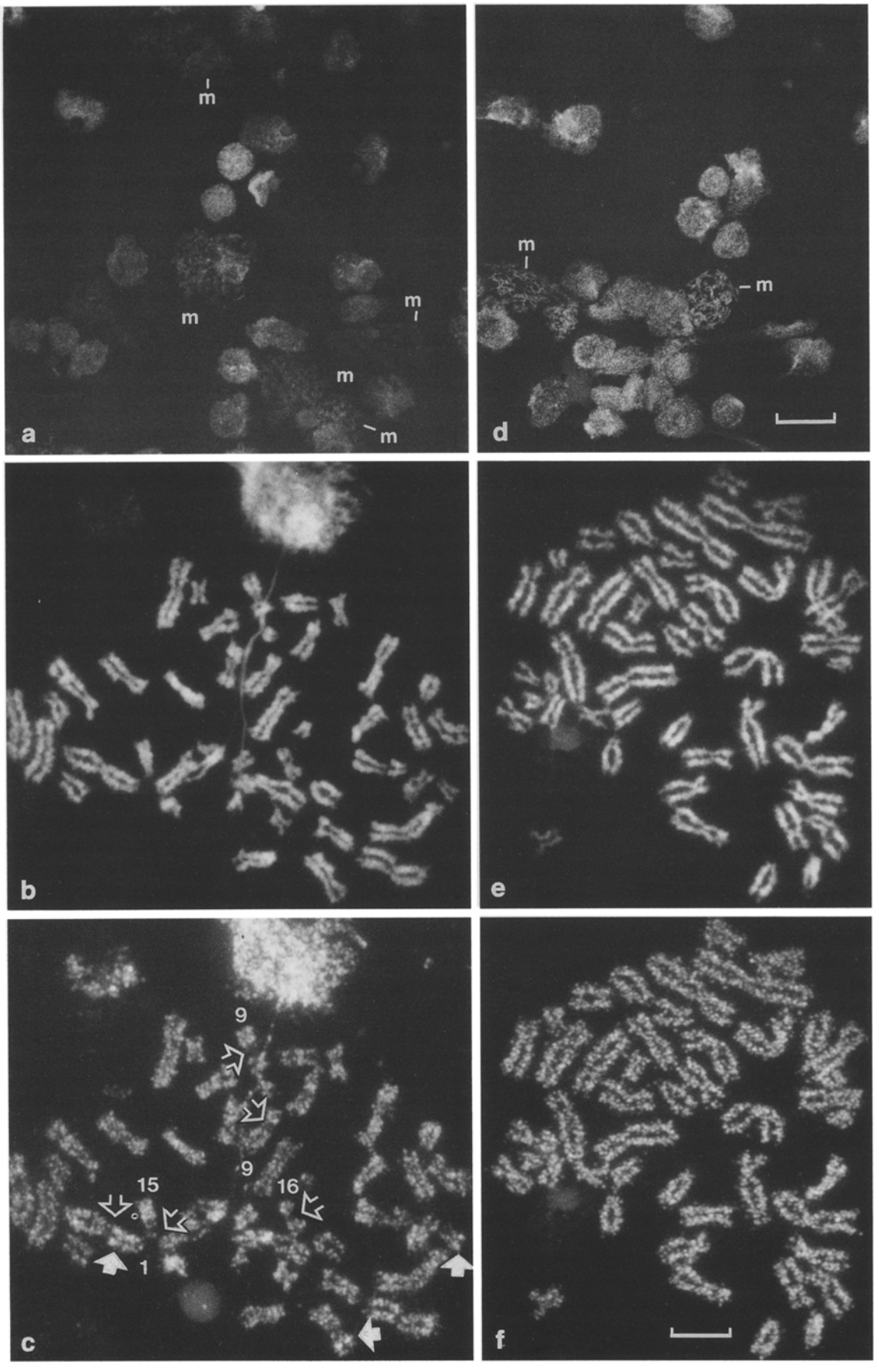
Rabbit serum R5/12

Serum R5/12 binds preferentially to histone H4 acetylated at lysine-12 (Turner and Fellows 1989). Figure 2 shows, by immunofluorescence, the binding of R5/12 (Fig. 2a, c) to human cell preparations, and compares this with another rabbit serum, R15/0 (Fig. 2d, f), that binds to non-acetylated, mono- and di-acetylated H4 isoforms (Turner et al. 1989). Individual cells vary in their intensity of immunofluorescence with R5/12, but in general, labelling of metaphase chromosomes is much less intense than labelling of interphase nuclei, although some metaphases do show a higher level of fluorescence. This is illustrated in Fig. 2a, where some of the metaphases are barely discernible, but one, located near the centre, has a significantly higher degree of labelling. At higher resolution, in a similar cell (Fig. 2c), the immunofluorescence is shown to be localized at a number of sites along the chromosome arms. This represents R5/12 binding to specific chromosomal locations, as evidenced by the corresponding fluorescence patterns on sister chromatids. In contrast, R15/0 gives a more uniform level of fluorescence in both nuclei and chromosomes (Fig. 2d), with chromosomes showing fairly homogeneous immunofluorescence (Fig. 2f).

Interestingly, the heterochromatic domains in chromosomes 1, 9 and 16 appear completely unlabelled by R5/12. This can be seen in normally condensed chromosomes (Fig. 2c), but was more evident when R5/12 was used to label undercondensed chromosomes containing stretched centromeres. Figure 3a shows a metaphase prepared from cells cultured in the presence of 5-azacytidine, indicating three chromosomes with undercondensed heterochromatic domains. In Fig. 3b this undercondensed heterochromatin is clearly seen to be unlabelled by R5/12. Immunofluorescence with R15/0, however, is continuous through the heterochromatic domains, and not significantly different when the heterochromatin is undercondensed (Fig. 3d).

The generally weaker fluorescence of metaphase chromosomes observed using R5/12, including the virtual absence in the centromeric heterochromatic domains, could be explained by a corresponding reduction in the accessibility of antibody to its binding site, due either to overall chromatin compaction, or to specific "burying" of the acetylated Lys-12 determinant within the conformation of H4. Since other anti-histone antibodies, including R15/0, HBC-7 and Ra120d (see below), produce quite strong immunofluorescence, either uniformly or with a different characteristic pattern (HBC-7), overall compaction of chromatin does not seem to be an adequate explanation for the reduced immunofluorescence seen with R5/12. As regards access to the actual epitope recognized by R5/12, studies involving tryptic digestion of whole avian erythrocyte chromatin have shown that the N-terminal regions of all four core

Fig. 2a–f. Histone H4 acetylation in human metaphase chromosomes detected by indirect immunofluorescence using antiserum R5/12. **a** Low magnification photomicrograph showing variation in intensity of FITC immunofluorescence exhibited by different cells with R5/12. In general, metaphases (*m*) show weaker fluorescence than interphase nuclei, although the metaphase in the centre of the field is as intensely labelled as the weaker nuclei. **b** Normal metaphase (Hoechst 33258, UV fluorescence). **c** Same metaphase as **b** showing R5/12 binding to H4 acetylated at Lys-12 detected by indirect FITC immunofluorescence. *Unfilled arrows* indicate the lack of labelling at centromeres of chromosomes containing major heterochromtic domains (identified). The *solid arrows* point to typical examples of chromosomes exhibiting bright punctate fluorescence patterns repeated on both chromatids. **d** Low magnification photomicrograph to show similar intensities of immunofluorescence of metaphases (*m*) and nuclei using R15/0. **e** Normal metaphase (Hoechst 33258, UV fluorescence). **f** Same metaphase as **e** showing uniform binding of serum R15/0 against non-acetylated H4, detected by immunofluorescence. Bars represent 30 μm (**a**, **d**) and 5 μm (**b**, **c**, **e**, **f**)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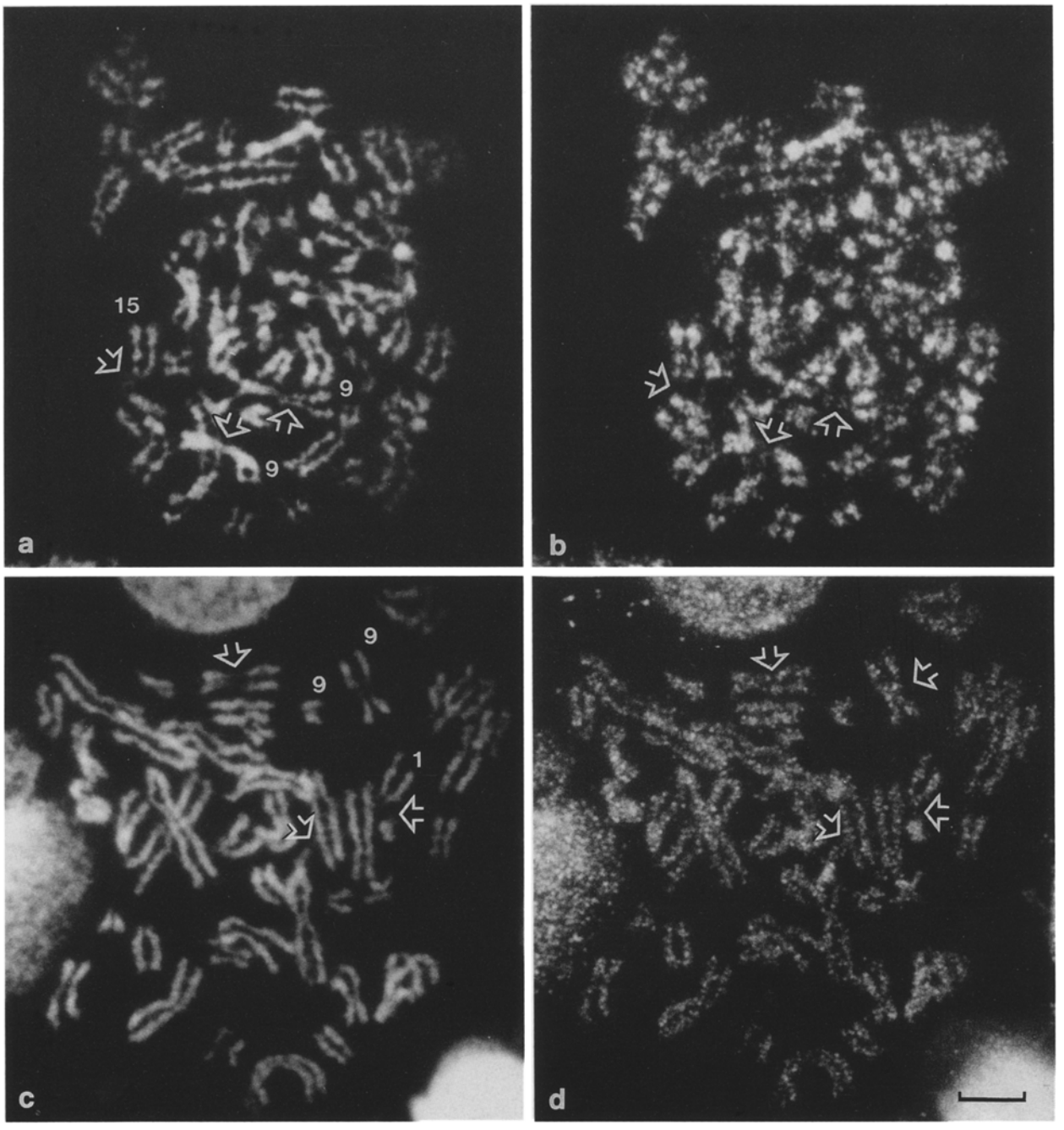

Fig. 3a–d. Reduced acetylation of histone H4 in major heterochromatic domains shown by low level of immunofluorescence using serum R5/12 on undercondensed chromosomes. **a** Human metaphase from lymphocyte culture grown in the presence of 5-azacytidine, indicating undercondensed heterochromatic domains (probable identities of chromosomes given) (Hoechst 33258, UV fluorescence). **b** Same metaphase as **a** showing serum R5/12 binding detected by indirect FITC immunofluorescence. Lack of binding in undercondensed heterochromatin is indicated by the *arrows*. **c** Human metaphase prepared from 5-azacytidine treated cell culture, indicating chromosomes containing undercondensed heterochromatic domains (Hoechst 33258, UV fluorescence). **d** Same metaphase as **c** showing binding of serum R15/0 to non-acetylated histone H4, detected by indirect FITC immunofluorescence. *Arrows* indicate continuity of fluorescent labelling through undercondensed heterochromatic domains. Bar represents 5 μm

histones are accessible (Weintraub and van Lente 1974), although this might not be the case at metaphase and/or in heterochromatin. However, the fact that R15/0 binds uniformly to metaphase chromosomes confirms that the N-terminal region of H4, which is specifically recognized by R15/0 (Turner et al. 1989), is accessible throughout metaphase chromosomes. Although we cannot exclude the possibility that the Lys-12 position itself is buried in the conformation of H4, or masked in some other way, to different extents at different locations along the chromosomes, we believe it to be more likely that the pattern of immunofluorescence observed in metaphase chromosomes using serum R5/12 reflects the degree of H4 acetylation at position Lys-12.

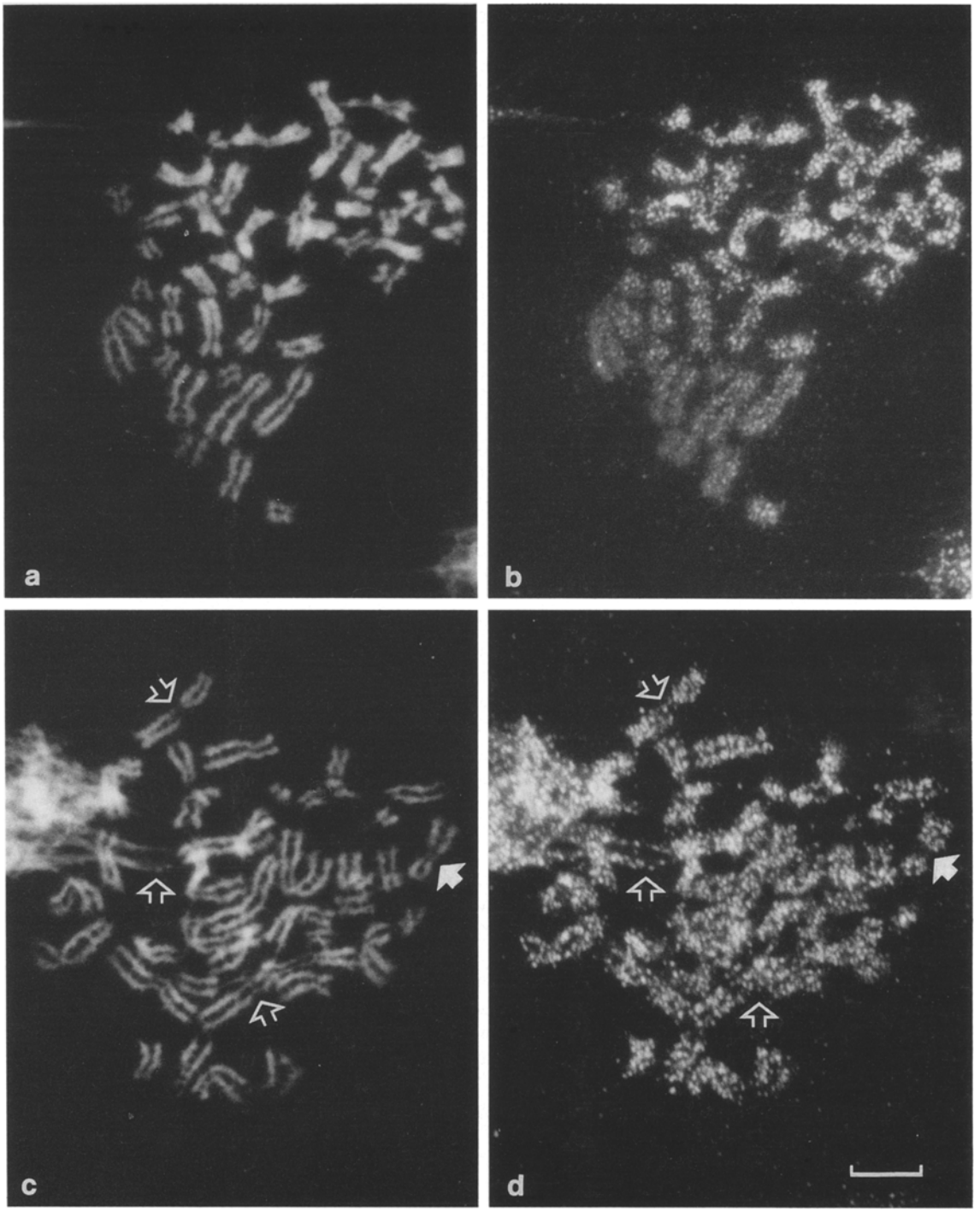

Fig. 4a–d. Binding of anti-histone H1 serum Ra120d to human metaphase chromosomes. **a** Normal metaphase (Hoechst 33258, UV fluorescence). **b** Same metaphase as **a** showing uniformity of binding of Ra120d, detected by FITC indirect immunofluorescence. **c** Metaphase prepared from lymphocyte culture treated with fluorodeoxyuridine (FUdR)/uridine procedure to induce undercondensation of heterochromatic domains. The *arrows* indicate undercondensed regions of unidentified chromosomes (Hoechst 33258, UV fluorescence). **d** Same metaphase as **c** demonstrating Ra120d binding detected by FITC indirect immunofluorescence. The *unfilled arrows* point to antibody binding through undercondensed regions. The *solid arrow* points to an apparently unlabelled undercondensed domain, such as was occasionally observed. Bar represents 5 μm

Rabbit serum Ra120d

Serum Ra120d was raised against calf histone H1 (Stollar and Ward 1970). H1 is the most loosely bound of the histones, and is also adjacent to the variable linker region in the chromatosome structure. It seemed possible, therefore, that differences in chromatin compaction, perhaps resulting from variation in nucleosome spacing, particularly within heterochromatin, might be reflected in differential accessibility to anti-H1 antibodies. It has

also been reported that in the α-satellite heterochromatin of the African green monkey, histone H1 is under-represented (Musich et al. 1977).

The results of indirect immunofluorescence in metaphase chromosomes using Ra120d are shown in Fig. 4. The FITC fluorescence has a more granular appearance than with the other antibodies described here, and is more diffuse than the corresponding Hoechst 33258 DNA fluorescence. This suggests that there had been some leaching of H1 from chromatin, and possible redistribution, under the conditions employed for the antibody reactions. Bearing in mind the loss of resolution that this may have introduced, the distribution of Ra120d binding appeared uniform, with no significant differences apparent in the heterochromatic domains of normal chromosomes (Fig. 4b). This was also true in the main for chromosomes treated to induce undercondensation of heterochromatin, although an occasional stretched chromosome appeared to be unlabelled in the heterochromatic domain. Examples of both labelling patterns are shown in Fig. 4d, for an FUdR/uridine treated metaphase.

Discussion

The immunofluorescence results presented here show clearly that binding of antisera to two specific histone determinants, namely the N-terminal region of H2B (monoclonal antibody HBC-7) and acetylated Lys-12 in H4 (serum R5/12) is nonuniform along human metaphase chromosomes. These antibodies also give nonuniform immunofluorescent labelling on isolated Chinese hamster chromosomes (Turner 1989). In similar experiments, using antibodies against whole histone fractions, we have confirmed that overall chromatin concentration does not vary appreciably, thus eliminating a possible explanation for our observations. We conclude that these results demonstrate that differences in histone modification and/or conformation exist at different locations along metaphase chromosomes. The techniques described in this report allow us to begin examining these differences in greater detail.

In most previous studies on histone acetylation in relation to the cell cycle (for example, D'Anna et al. 1983), acetylation patterns in total chromatin extracts have been used for comparisons. In this report we have been able to examine histone acetylation in situ. Serum R5/12 binding has been shown previously to be a good indicator of histone H4 hyper-acetylation (Turner and Fellows 1989): acetylation at Lys-12 is underrepresented in mono-acetylated H4 and occurs predominantly in di-tri- and tetra-acetylated isoforms. Hyper-acetylation of H4 has been associated with transcriptional activity, and consequently, R5/12 binding to chromatin might also be expected to correlate with transcriptional activity. Our results are consistent with this hypothesis, and with previously published data (D'Anna et al. 1983): thus, in general, we find that R5/12 binding to transcriptionally inactive mitotic chromosomes is less than to interphase nuclei, whereas serum R15/0, which recognizes non-acetylated, mono- and di-acetylated isoforms of H4 (Turner et al. 1989), binds both mitotic chromosomes and interphase nuclei approximately equally.

One interpretation of the immunofluorescence patterns observed in metaphase chromosomes using R5/12 is that the regions of brighter fluorescence indicate sites of late transcriptional activity, leading to more highly acetylated H4 isoforms surviving into the early stages of mitosis. By combining metaphase arrest with brief butyrate treatments to inhibit deacetylases, it has been shown that turnover of H4 acetate groups in Chinese hamster cells continues throughout metaphase (Turner 1989). However, the amounts of the more highly acetylated isoforms are dramatically reduced. As the experiments described here were carried out in the absence of butyrate, the fact that only certain metaphases showed significant labelling could be explained by these cells having been in metaphase arrest only briefly before harvesting. Those cells arrested in metaphase for longer periods would lose the more highly acetylated H4 isoforms, which is consistent with the generally reduced immunofluorescence of metaphase chromosomes compared with interphase nuclei that we observed with R5/12.

Preliminary results with antisera directed at other specific acetylation sites on H4 gave results qualitatively similar to those with R5/12, described above (unpublished observations). In particular, like R5/12, all antisera recognizing acetylated forms of H4 led to very low, or barely detectable immunofluorescence in the heterochromatic domains of chromosomes 1, 9 and 16. Histone H4, therefore, is clearly under-acetylated in these regions. We believe this to be the first demonstration of specific under-acetylation of histone in heterochromatin, at least in mitotic chromosomes. This may be related to the non-transcriptional role of heterochromatin, or to heterochromatin-specific structural factors.

The brighter indirect immunofluorescence of the heterochromatic domains observed using mouse monoclonal antibody HBC-7 shows that heterochromatin differs from euchromatin in another way, although the nature of the difference is not clear. This difference applies not only to the centromeric domains, but also to the Y chromosome heterochromatin (Fig. 1b). Enhanced binding of HBC-7 to histone H2B in heterochromatin, resulting in increased immunofluorescence, could be due to a conformation difference, allowing greater access of antibody to its binding site in heterochromatin compared with euchromatin. HBC-7 is an IgM recognizing an epitope within the N-terminal eight residues of H2B (Whitfield et al. 1986). Differences in overall compaction of heterochromatin, or local changes in conformation of H2B and/or nucleosomes could be envisaged as altering accessibility of the large antibody molecule to its epitope. Intercalation of ethidium bromide into DNA contained in nucleosomes and chromatin has been shown to increase the binding of HBC-7 several fold (Whitfield et al. 1986), as have nuclease digestion and exposure to high salt buffers (Turner 1982). These treatments presumably exert their effects on antibody binding through changes in nucleosome conformation.

An alternative explanation is that modifications to histone H2B modulate antibody binding differently in the two forms of chromatin. The eight residue N-terminal region of H2B, which incorporates the HBC-7 recognition site, contains three known modification positions: an ADP-ribosylation site at Glu-2, an acetylation site at Lys-5, and a phosphorylation site at Ser-6 (Wu et al. 1986). It is not known whether acetylation or phosphorylation at these positions affect HBC-7 binding, but ADP-ribosylation of Glu-2 abolishes antibody recognition (Whitfield et al. 1986). However, only about 2% of total H2B occurs as the ADP-ribosylated derivative (Matthews 1988), so it is unlikely that the enhanced HBC-7 binding to heterochromatin is the result of a complete absence of this modification. The function of ADP-ribosylation is obscure.

Acetylation of histone H2B is thought to be confined to Type I (very low turnover rate of no known function) and Type III (short-lived, associated with synthesis and chromatin assembly) (Matthews 1988). Type III acetylation would not be expected to persist into metaphase, so it is unlikely to be involved in modulating HBC-7 binding, but Type I acetylation could differ between heterochromatin and euchromatin. Very little is known about phosphorylation of H2B, except that it occurs. The other histone modifications may be discounted: ubiquitination of H2B occurs in the C-terminal portion, and methylation of H2B has not been described.

The major heterochromatic domains are distinguished by their differential labelling with antibodies to defined histone epitopes, as demonstrated here, by their distinctive staining with a number of dyes and fluorochromes, and by their tendency to decondense under a variety of treatments. These properties suggest that the blocks of simple-sequence satellite DNAs contained within the heterochromatic domains share a unique chromatin packaging arrangement. Two satellites, 2 and 3, are related, with their repeat structures based on the pentameric sequence CCATT (Prosser et al. 1986). Satellite 2 is common to all of the domains, whereas satellite 3 is confined to chromosomes 9, 15 and the Y (Gosden et al. 1975). It seems likely that the unique structure of centric heterochromatin involves as yet unidentified nonhistone proteins that recognize specific DNA sequences. Heterochromatin specific proteins have been recognized in mouse (Haaf et al. 1990), African green monkey (Strauss and Varshavsky 1984), and in *Drosophila* there is good evidence for the existence of heterochromatin-forming proteins (e.g. Reuter et al. 1990; James and Elgin 1986; Gatti et al. 1983).

A number of different procedures have been described that result in undercondensation of the major heterochromatic domains in the human karyotype, although the mechanisms are not yet understood. The presence in the culture medium of non-intercalating, DNA-binding drugs such as Hoechst 33258 (Hilwig and Gropp 1973) and DAPI (Rocchi et al. 1979) represents one group of such treatments, while growth with cytidine analogues such as 5-azacytidine and 5-azadeoxycytidine forms another (Schmid et al. 1983). For the latter group, there is evidence that the cytosine-analogous base is incorporated into DNA, inhibiting DNA methylation (Jones and Taylor 1980), and it has been postulated that the undercondensation of heterochromatin results from an undermethylation of the corresponding DNA (Schmid et al. 1983). There is some evidence for enrichment of 5-methylcytosine in the heterochromatic domains (Miller et al. 1974). Undercondensation of the heterochromatin would result if the binding of heterochromatin-forming protein to DNA were inhibited by, for example, the presence of DNA ligands such as DAPI, or by incorporated cytosine analogues. (This latter scenario could indicate that the putative protein recognizes methylated DNA sequences.)

We have shown in this report that the major heterochromatic domains differ from the bulk of euchromatin in their binding to the specific anti-histone antibodies R5/12 and HBC-7. In the former case we have argued that the difference resides in under-acetylation of histone H4 in the heterochromatic domains. The enhanced binding of HBC-7 to the N-terminal region of histone H2B in normal heterochromatin can be explained by increased accessibility as a result of a conformation difference, or to the lack of some unidentified modification. The observation that there is no enhancement of HBC-7 binding to decondensed heterochromatin, following the varied treatments described here to induce undercondensation, suggests to us that a conformational difference is responsible for the enhancement seen in normal chromosomes, although this point requires further clarification. Nevertheless, it is likely that the conformation adopted by heterochromatin at the nucleosome level, possibly under the influence of nonhistone protein as postulated above, is intimately linked to the presence or absence of core histone modifications. This could either be direct, with the histone modification state helping to maintain the conformation, or as a secondary effect through conformation differences altering access to modifying and de-modifying enzymes.

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