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Differential immunostaining of plant chromosomes by antibodies recognizing acetylated histone H4 variants

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Metaphase chromosomes of *Vicia faba* were exposed to antibodies recognizing defined acetylated isoforms of histone H4. After indirect immunostaining with antibodies directed against H4 acetylated on lysines 5, 8 and 12 respectively, the entire chromosome complement was labelled. The brightest signal appeared at the nucleolus organizing region (NOR). The large genetically inert heterochromatic regions, which are composed of late replicating tandemly repetitive DNA sequences, remained unlabelled. Thus, the chromosomal distribution of histones H4 acetylated at positions of lysine 5, 8 and 12 is broadly correlated with the intensity of transcription and the sequence of replication of the field bean chromatin during interphase. Antibodies against H4 acetylated at lysine 16 also caused a strong signal at the NOR but otherwise a uniform fluorescence along the chromosome.

Key words: acetylated histone H4, heterochromatin, indirect immunofluorescence, nucleolus organizing region, *Vicia faba*

Introduction

The histones H2A, H2B, H3 and H4 are very conservative proteins. As an octamer they form the nucleosome cores of eukaryotic nuclear chromatin (for review see Felsenfeld 1978). Although their structural importance is known, their role in transcription, replication and repair processes is just recently becoming clearer. All core histones are subject to post-translational modifications such as phosphorylation, ADP ribosylation, methylation and acetylation (Matthews 1988). The acetylation of the N-terminal lysine residues of the core histones has been a subject of particular interest not least due to its frequent association with transcription first noted by Allfrey *et al.* (1964) and recently reviewed (Turner 1991, 1993). Reversible acetylation of the histones is mediated by chromatin-associated acetyltransferase/deacetylase enzymes (Attisano & Lewis 1990) and is generally believed to play a role in modulating the higher order folding of chromatin (Loidl 1988, 1994). In most cases studied so far H4 lysines 5, 8, 12 and 16 become acetylated in a certain order (Turner 1991). For example, in mammals, amphibia and yeast lysine 16 is the first site of acetylation.

In the pericentric heterochromatin of mammals, H4 is underacetylated at all four N-terminal lysines (Jeppesen *et al.* 1992). The same is true for the inactive X chromosomes of female mammals, whereas the R-band regions, which are enriched in coding sequences, revealed a relatively high level of H4 acetylation (Jeppesen & Turner 1993).

In *Drosophila* polytene chromosomes, H4 is underacetylated at all sites except lysine 12 in centric heterochromatin and preferentially acetylated at lysine 16 in the X chromosome of males (Turner *et al.* 1992). Genes on the single male X chromosome are transcribed twice as rapidly as those on the two female X chromosomes and the acetylation of the H4 at lysine 16 is likely to be the integral component of their dosage compensation mechanism (Bone *et al.* 1994). Thus, both the overall level of acetylation and acetylation of individual lysines are used to define functionally distinct chromosomal domains.

The H4 N-terminal sequence in plants is the same as in mammals (Smith *et al.* 1995) and can also be the subject of acetylation, although the order of acetylation of different lysine residues is as yet unknown. In the present study we applied immunolabelling with antibodies against histone H4 acetylated on lysines 5, 8, 12 and 16 to the cytologically well-characterized chromosomes of the field bean *Vicia faba*. The labelling patterns observed proved to be similar to those reported for mammalian and insect chromosomes.

Materials and methods

Antibodies

The antibodies used were polyclonal rabbit antisera, which recognized specifically histone H4 acetylated at lysine positions 5 (R41/5), 8 (R12/8), 12 (R20/12), 16 (R14/16) and non-acetylated H4 (RSU) respectively (Turner & Fellows, 1989, Turner *et al.* 1989). As controls, monoclonal anti-DNA antibodies (Boehringer) directed against single- and double-stranded DNA were used.

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Chromosome preparation and indirect immunofluorescence A pure suspension of metaphase chromosomes and interphase nuclei was prepared from synchronized root meristems of seedlings of a field bean line with a reconstructed karyotype (ACB), characterized by individually distinguishable chromosome pairs, according to Schubert *et al.* (1993). Indirect im-

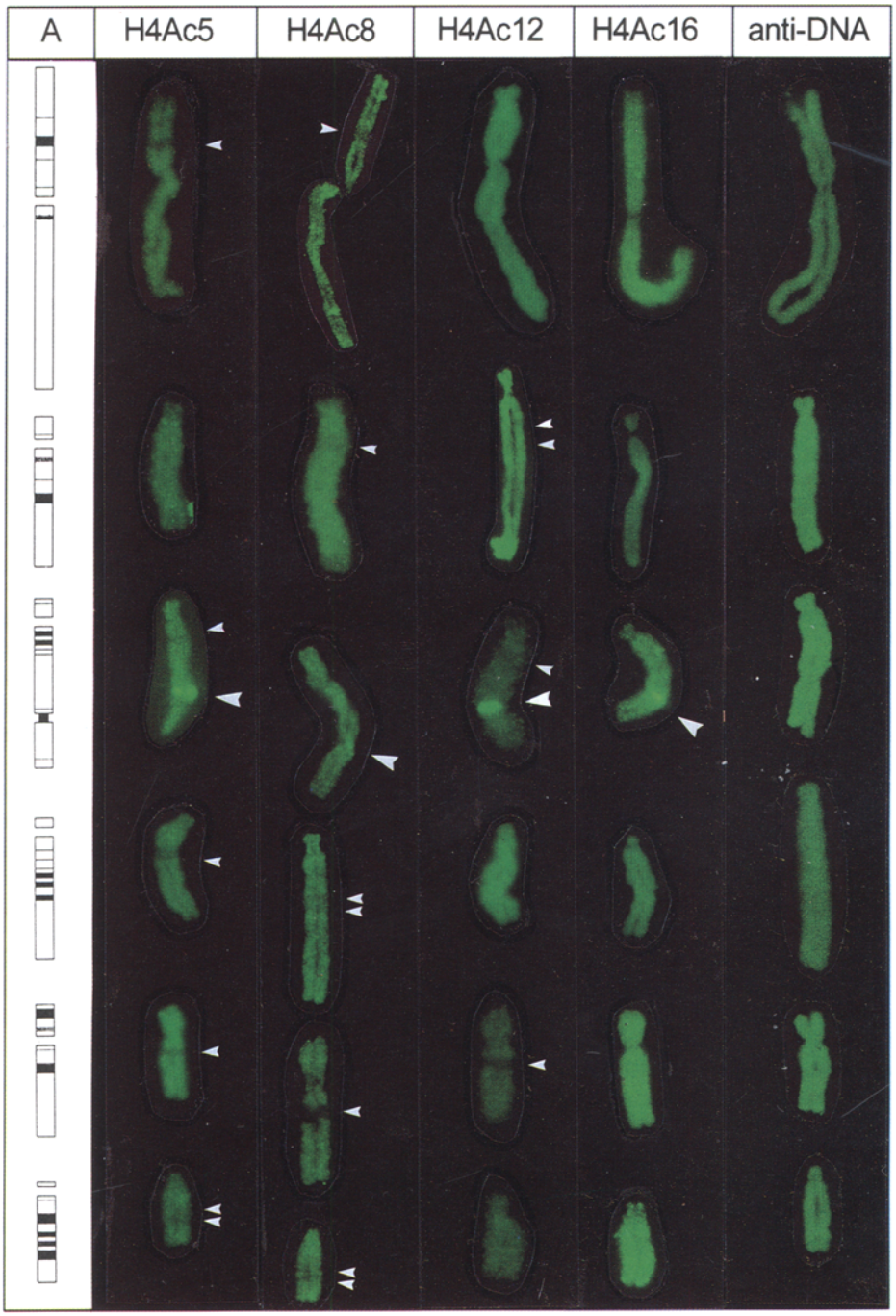

Figure 1. Chromosomes of the field bean karyotype ACB. Scheme of Giemsa-banded chromosomes (A); indirect immunofluorescence mediated by antibodies against histone H4 acetylated at position of lysine 5 (H4Ac5), lysine 8 (H4Ac8), lysine 12 (H4Ac12) and lysine 16 (H4Ac16), and against DNA. Bright fluorescence at the NOR is marked by large arrowheads, underacetylated late-replicating heterochromatin (sometimes difficult to reproduce photographically) by small arrowheads.

munofluorescence was done as described by Houben *et al.* (1995). The suspension was dropped on slides and covered with a coverslip. After freezing in liquid nitrogen, the coverslips were removed and the slides transferred immediately into phosphate-buffered saline (PBS, pH 7.3). Then, chromosomes were incubated for 1 h at 4°C in primary sera (diluted 1:400 in PBS) in a humidified chamber, washed in PBS and incubated for 1 h at 4°C with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Dianova), diluted 1:40 in PBS. In the case of the anti-DNA antibody, FITC-conjugated anti-mouse IgG (Dianova) was used. Following a further three washes in PBS, the preparation were post-fixed for 10 min in PBS containing 4% (v/v) formaldehyde. Fluorescently labelled chromosomes were observed using a microscope equipped with epifluorescence optics. The photographs were taken on Kodak Ektachrom 400 films.

Results and discussion

Specific antibodies were used for the first time to study the distribution of acetylated variants of histone H4 in mitotic plant chromosomes. All four antibodies directed against H4 acetylated at lysine position 5, 8, 12 or 16 labelled the entire chromosome complement of the field bean. The brightest fluorescence was observable at the nucleolus organizing region (NOR). The antibodies against H4Ac16 labelled the remaining chromosome parts uniformly. The antibodies against H4Ac5, H4Ac8 and H4Ac12 left the most extended interstitial heterochromatic regions of chromosomes I, II, III, IV, V and VI unlabelled (Figure 1). This is most easily observed on less condensed prometaphase chromosomes. Interphase nuclei showed diffuse labelling after treatment with each of the four antibodies. Anti-DNA antibodies yielded uniform labelling along all chromosomes. The same was true for antibodies against non-acetylated H4 (data not shown). This indicates a basically uniform distribution of non-acetylated H4 independent of additional hyperacetylation, as for example at the NOR. The preimmune sera used as a control did not stain the chromosomes.

The correlation of this labelling pattern with the sequence of replication and the potential transcription activity is remarkable. The single 18/25S rDNA locus of the field bean not only reveals highest transcriptional activity but is also the earliest replicating region of the genome (Schubert & Rieger 1979). The large heterochromatic regions in midarm position, which seemed to be depleted of H4Ac5, H4Ac8 and H4Ac12, coincide positionally with so-called Giemsa marker bands and late-replicating regions (Döbel *et al.* 1978, Schubert & Rieger 1991) and consist mainly or exclusively of tandemly repeated sequence elements (Fuchs *et al.* 1994). When hybridized *in situ* with labelled total cDNA pooled from all organs of the species, again these regions are the only ones showing no or less dense signals (Houben *et al.* 1994).

Our results are generally similar to those reported for animal cells in so far as the NOR, which is heavily transcribed, showed the most pronounced signal with all four antibodies and three of the acetylated isoforms are apparently absent from the heterochromatic regions that lack coding DNA and are not active in transcription.

The observation that H4Ac5, H4Ac8 and H4Ac12 are absent from the inactive heterochromatin, while H4Ac16 is present, can be interpreted as showing that heterochromatin is depleted in hyperacetylated H4 but retains the monoacetylated isoform H4Ac16, assuming the same order of H4 acetylation for plants and vertebrates.

It is tempting to speculate that H4Ac16 might have a specific function in the formation of heterochromatin in plants similar to H4Ac12 in *Drosophila* as these are the only lysine positions that remain acetylated in the otherwise underacetylated heterochromatin.

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