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Tomographic distribution of acetylated histone H4 in plant chromosomes, nuclei and nucleoli

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Abstract. Root tip cells of broad bean (*Vicia faba* L. cv. 'Wase soramame') and barley (*Hordeum vulgare* L. cv. 'Minorimugi') were immunostained with antibodies specific for acetylated histone H4. With an antiserum that recognizes histone H4 acetylated at lysine-5, the nucleolar organizing region (NOR) in mitotic chromosomes was strongly labeled in both species. The broad bean had two signals in the metaphase and telophase chromosome complements and four signals in the prophase and anaphase chromosome complements, while the barley had four signals in the metaphase and telophase chromosome complements and eight signals in the prophase and anaphase complements. Five different patterns of signals were observed at interphase: in type I only nucleoli were wholly stained; in type II perinucleolar knob-like signals and/or fiber-like signals emanated from the nucleus; in type III aggregate signals appeared in the nucleolus; in type IV many small dot-like signals were distributed throughout the nucleus, except nucleoli; and in type V string-like or some granule-like signals appeared in the nucleoli. Type II was very similar to previous results by in situ hybridization with sense rDNA probes. Type III was similar to the patterns of DNA synthesis recognized as chromatin domains by anti-BrdU antibodies. Type V was very similar to the results of in situ hybridization with pTa71, rDNA probes and the appearance of the dense fibrillar components of the nucleolus.

Introduction

Eukaryotic cells have evolved cell-cycle-dependent processes that modulate histone: DNA interactions in chromosomes. These include: (1) acetylation of selected lysines on the four core histones; (2) phosphorylation of serines and threonines on core and linker histones; and (3) ubiquitination. Histone acetylation has been associated with genome replication and transcription (Bradbury 1992). In the case of histone H4, there are four lysine residues that can be reversibly acetylated. Turner et al. (1989) have developed antibodies that distinguish H4 molecules acetylated at each of the sites used in vivo. They characterized the specificity of each antiserum for individual acetylated sites by measuring the ability of synthetic peptides to inhibit antibody binding to histones in a solid-phase immunoassay. They showed each serum contained antibodies directed against a single acetylated site and narrowly defined epitope. Because of the extreme evolutionary conservation of H4, these antisera can be applied to a wide variety of organisms and experimental systems. Turner et al. (1990) used antisera to histone H4 acetylated at Lys-5 (R6/5) and Lys-12 (R5/12) to label polytene chromosomes in *Chironomus* salivary gland nuclei. They concluded that, in general, levels of acetylation at lysine 5 and 12 vary in parallel. The nucleolar organizing region was strongly labeled by both antibodies. Turner et al. (1992) tried antisera to histone H4 acetylated at Lys-5 (R6/5), Lys-8 (R12/8), Lys-12 (R20/12) and Lys-16 (R14/16) against polytene chromosomes in *Drosophila* salivary gland nuclei. H4Ac16 was found predominantly in the transcriptionally hyperactive dosage-compensated X chromosome of male larvae. H4 acetylation at Lys-16 has been shown to provide a consistent cytogenetic marker for dosage compensation (Steinemann et al. 1996). H4Ac5 and H4Ac8 were found in sharply defined, overlapping but nonidentical islands dispersed through all four chromosomes. Relatively low levels of H4 acetylated at these sites were found in the β-heterochromatin of the chromocenter. In contrast, H4Ac12 occurred in heterochromatin at a level equal to or greater than that found elsewhere in the genome. Jeppesen and Turner (1993) tried four antisera, R6/5, R12/8, R5/12 and R13/16, against mouse and human metaphase chromosomes. Antisera recognizing H4 acetylated at lysine-5, lysine-8, and lysine-12, all gave rise to R-like banding patterns, with antiserum against lysine-5 producing the highest differentiation between bands and interbands. Banding with antiserum against

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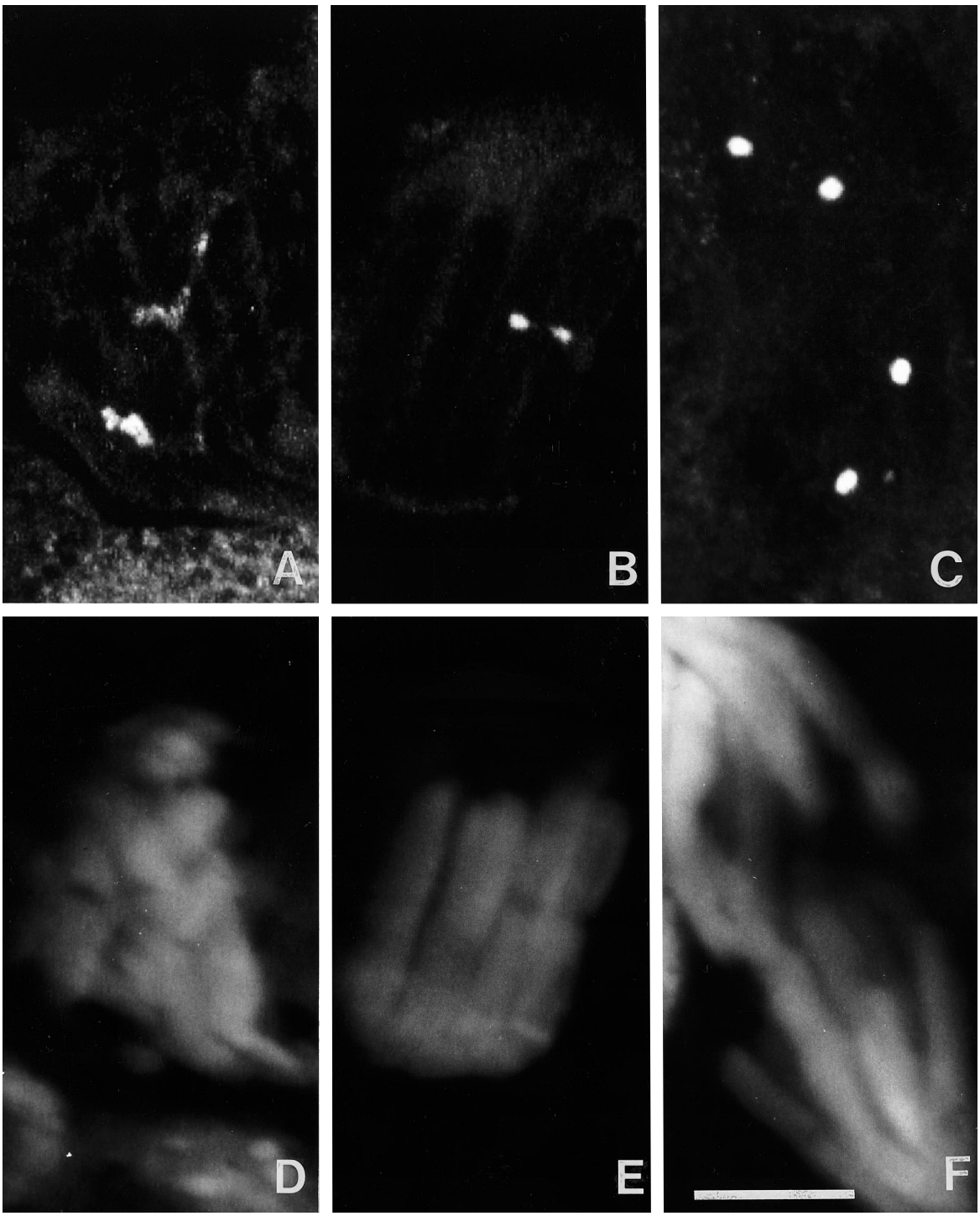

Fig. 1A–F. Labeling of histone H4 acetylated at lysine-5 in chromosomes of *Vicia faba.* The preparations were made by the enzyme maceration squashing method and examined by the confocal laser scanning microscope. Chromosomes at prometaphase (**A, D**), metaphase (**B, E**), and anaphase (**C, F**). **A–C** FITC signals. **D–F** DAPI staining. Bar=10 μm

H4 acetylated at lysine-16 was the least clearly defined. Centric heterochromatin and the inactive X in female cells were unlabeled with all antisera.

In plants, the labeling patterns with antibodies against histone H4 acetylated on lysines 5, 8, 12 and 16 proved to be broadly similar to those reported for mammalian and insect chromosomes. Houben et al. (1996) tried four antisera that recognized specifically histone H4 acetylated at lysine positions 5 (R41/5), 8 (R12/8), 12 (R20/12) and 16 (R14/16) in metaphase chromosomes of *Vicia faba*. With antisera to H4 acetylated on lysine 5, 8, and 12, respectively, the entire chromosome complement was labeled. The brightest signal appeared at the nucleolus organizing region. The large genetically inert heterochro-

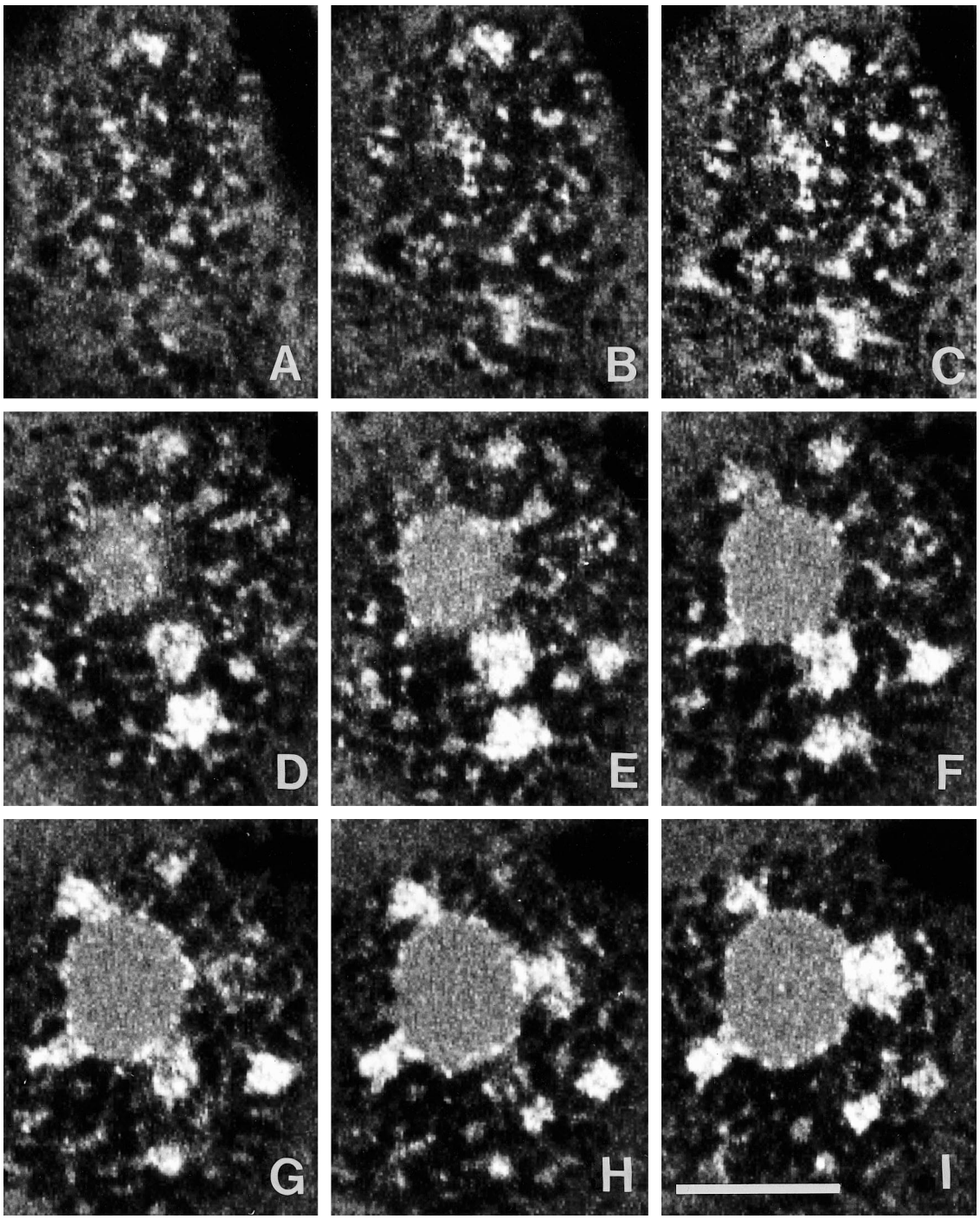

Fig. 2A–I. Nine confocal optical sections of a *Vicia faba* root nucleus labeled with antibodies against histone H4 acetylated at lysine-5. Sections were selected from a stack of 99 sections at a 0.1 µm interval through the z-axis and projected into two dimensions. Sections from the bottom of the cell upwards are shown left to right. In the first two pictures, fiber-like structures are clearly visible. The perinucleolar labeled sites are prominent and small spots emanate from some of these into the body of the nucleolus (type II). The boundary of the nucleus is outlined by faint cytoplasmic fluorescence and the nucleolus by faint fluorescence visible in the middle sections. **A** Projected result of the first 11 sections. **B–I** Projected results of the next eight groups of 11 sections. Bar=10 µm

matic regions that were composed of late replicating, tandemly repetitive DNA sequences remained unlabeled. Antibodies against H4 acetylated at lysine 16 also caused a strong signal at the NOR, but otherwise a uniform fluorescence along the chromosomes. They discussed the chromosomal distribution of histone H4 acetylated at positions 5, 8 and 12, which was broadly correlated with the intensity of transcription and the sequence of replication of field bean chromatin during interphase. However, they examined only metaphase chromosomes. Thus, we applied one of the same antibodies (R41/5) and analyzed the distribution patterns three-dimensionally during each

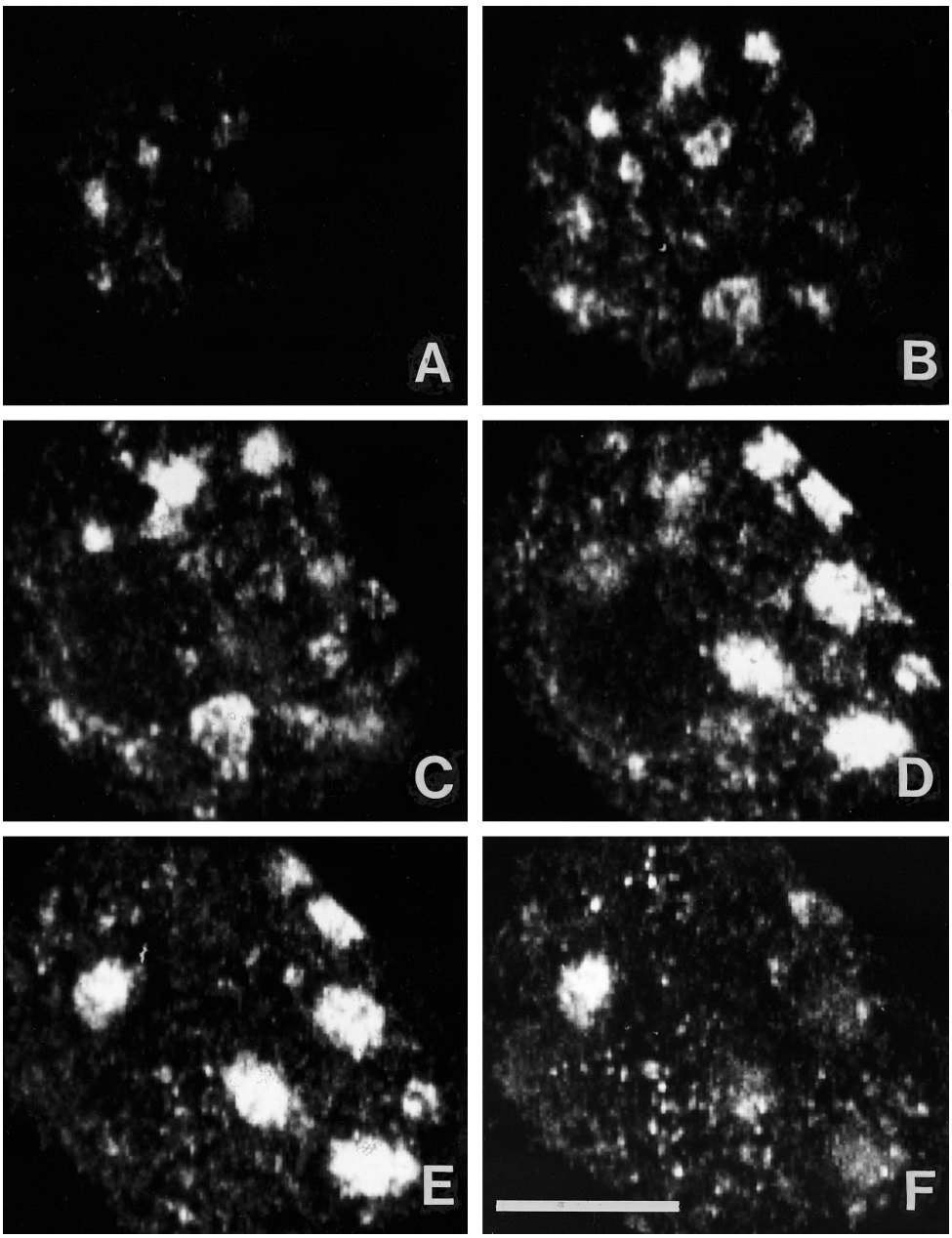

Fig. 3A–F. Six confocal optical sections of *Vicia faba* root nucleus labeled with antibodies against histone H4 acetylated at lysine-5 and made by the confocal laser scanning microscope. Sections were selected from a stack of 54 sections, each of which was obtained at a 0.1 µm interval through the z-axis, and projected into two dimensions every ninth stack. Aggregate signals (type III) in the nucleus were visible. **A** Projected result of the first nine sections. **B–F** Projected results of the next five groups of nine sections each. Bar=10 µm

mitotic phase and interphase. Additionally, we compared the signal patterns in the broad bean and barley.

Materials and methods

Plant materials. Seeds of broad bean (*Vicia faba* L. cv. 'Wase soramame') were purchased from a commercial source, and those of barley (*Hordeum vulgare* L. cv. 'Minorimugi') were a gift from the Hokuriku National Agricultural Experiment Station. They were sown on wet filter paper in petri dishes at 24° C for the broad bean and 30° C for barley.

Preparation. Their root tips (3–10 mm long) were cut off and fixed with potassium chromosome medium (KCM; Gooderham and Jeppesen 1983) containing 1% triton X-100 and 2% formalin at 0° C for 10 min in KCM. Subsequently, the procedure of Terasaka and Niitsu (1994) was followed with a little modification. The root tips were macerated in a humid chamber at room temperature for 40 min. Then the tissue were squashed through coverslips coated with 1% poly-L-lysine. The slides were air dried for 3 min and then washed again with KCM for 5 min and immunostained.

Antibodies. The R41/5 serum was diluted 500-fold in KCM containing 1% normal goat serum. The secondary antibody, anti-rabbit IgG FITC conjugate (Sigma), was diluted 200-fold with KCM containing 10% goat serum.

Immunostaining. The slides were incubated with primary antibody for 60 min in a humid chamber at room temperature, followed by washing with KCM in a petri dish three times for 10 min each. Secondary antibody incubation was for 30 min in a humid chamber at room temperature, followed by three washes in KCM as after the primary antibody incubation. 4´,6-Diamidino-2-phenylindole (DAPI) (2 µg/ml) was used for counterstaining. The slides

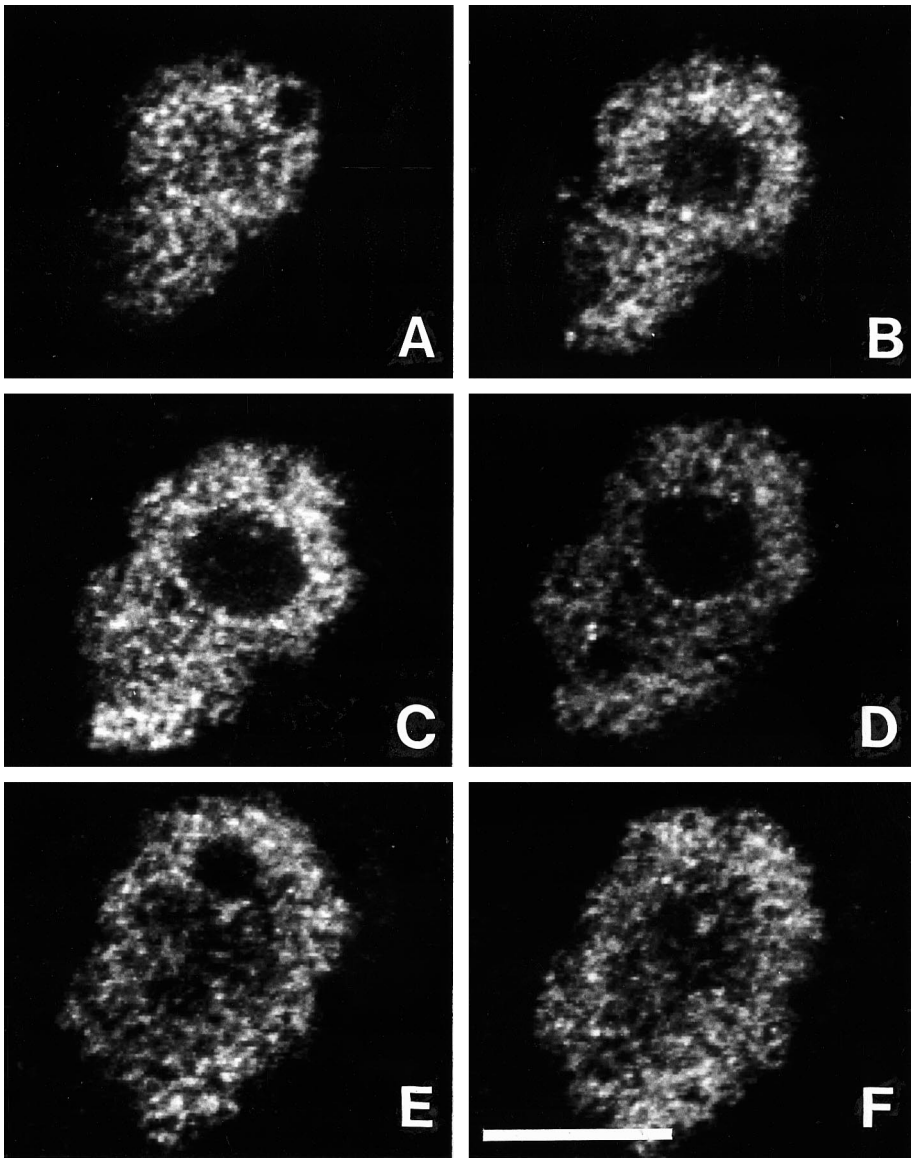

Fig. 4A–F. Six confocal optical sections of *Vicia faba* root nucleus labeled with antibodies against histone H4 acetylated at lysine-5. Sections were selected from a stack of 48 sections, each obtained at a 0.1 µm interval through the z-axis, and transparented into two dimensions every eight stacks. Small dot-like signals (type IV) were visible at each image. Dots appeared throughout the nucleus without nucleoli. Those distribution patterns were correlated with the DAPI staining pattern. **A** Projected result of the first eight sections. **B–F** Projected results of the next five groups of eight sections each. Bar=10 µm

were mounted in glycerol containing 1% triethylene diamine (DABCO) and sealed by nail polish. After storage overnight at 5° C to allow the antifade solution to penetrate, the slides were examined with fluorescence microscopy (Axiophot FI; Carl Zeiss, Germany) and confocal laser-scanning microscopy (LSM 410 UV; Carl Zeiss, Germany).

Three-dimensional observation. Slides were analyzed using the confocal laser scanning microscope with an argon ion laser at 488 nm wave length and ultraviolet laser at 396 nm wavelength. Images of antibody-FITC signals were obtained consecutively by mechanically shifting with 0.1 µm steps along the z-axis and three dimensionally analyzed. The DAPI images were obtained consecutively by mechanically shifting with 0.5 µm steps along the z-axis. Images were obtained using 100 X plan neofluar objective lens and 1.6 times enlarged in the computer and stored in magneto optical disks.

Results

1. Broad bean Vicia faba cv. 'Wase soramame'

In mitotic chromosomes, FITC signals were observed at the secondary constriction sites that were recognized as negative regions of DAPI counterstaining (Fig. 1). Two prominent FITC signals were observed in the metaphase and telophase chromosome complements and four in the prophase and anaphase chromosome complements. Prophase signals showed a more extended appearance than other three stages.

At interphase, five different signal patterns were observed. In type I only the nucleoli were totally stained (Fig. 5A, B), and this type was the majority. A total of 369 out of 884 interphase nuclei studied in root tip cells labeled with anti-AcH4 antibody showed type I labeling. Nucleoli varied in diameter 3–8 µm. Condensed small nucleoli of differentiated cells showed this labeling pattern.

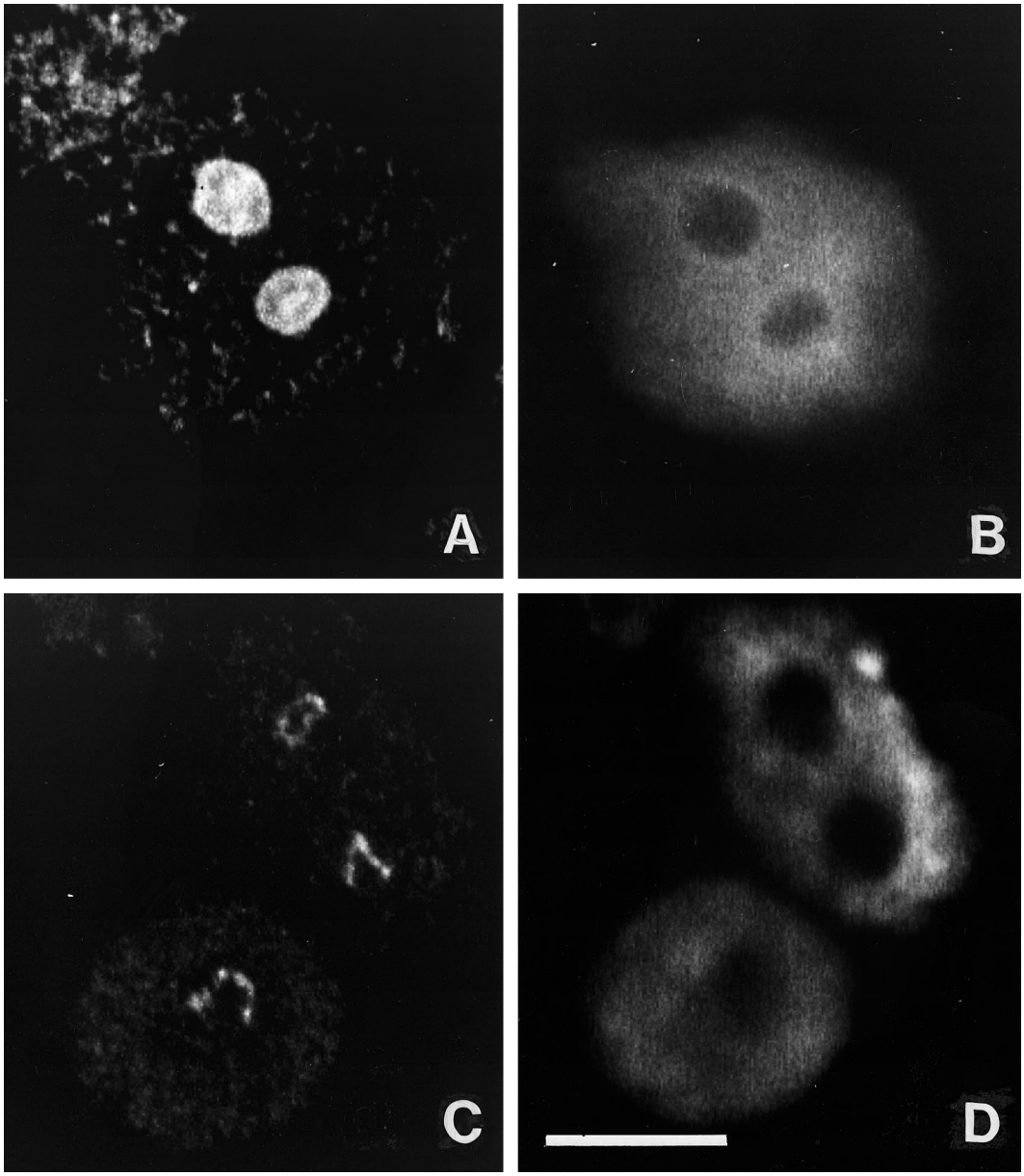

Fig. 5A–D. Signal patterns other than type II, type III and type IV with antibodies against histone H4 acetylated at lysine-5 in interphase nuclei of *Vicia faba*. **A**, **C** FITC signals. **A** Nucleolus was fully stained (type I). **C** String-like or granule-like signals (type V). **B**, **D** DAPI fluorescence in the same figure as in **A** and **C**. Bar=10 μm

Perinucleolar knob-like and/or fiber-like signals emanated from the nucleoli in some nuclei (Fig. 2, type II). Fiber-like signals are visible in the first two pictures. On the fiber networks here and there were observed some strong dot signals. Perinucleolar knob-like signals were very similar to those obtained by in situ labeling results with sense rDNA probes (Rawlins and Shaw 1990).

Some nuclei had many aggregate signals (Fig. 3, type III). About ten big, chromatin domain-like structures 2–5 μm in diameter and many small fragments were labeled. The big, prominent signals were not distributed equally throughout the nucleus. The chromatin domain-like conjugate signals showed polarized arrangement in the nucleus.

Some other nuclei had many small, dot-like signals (Fig. 4, type IV). Three-dimensional confocal observation showed the dot-like signals to be distributed throughout the nucleus except at the site of nucleoli and small spherical structures 1.5–3 μm diameter.

Figure 5C, D shows the signal pattern that specifically appeared in nuclei as string-like FITC signals, coincident with the site of nucleoli in DAPI images (type V). The nucleolus had two string-like signals when a nucleus had one nucleolus, but when a nucleus had two nucleoli, each of them had only one string. Some nuclei had granule-like signals in the nucleoli instead of string-like signals. The granule-like signals were considered to be string-like signals that had been fragmented.

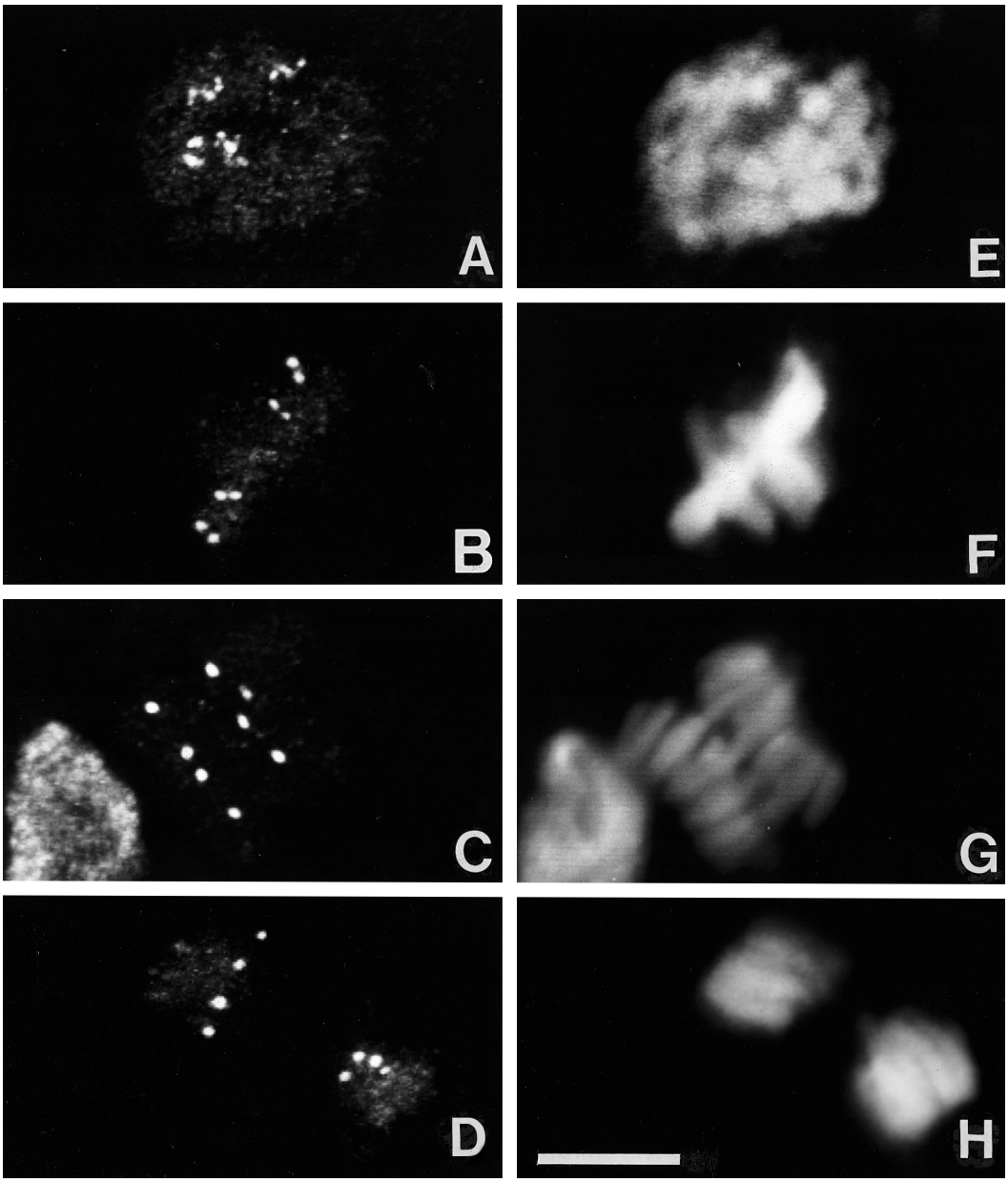

Fig. 6A–H. Labeling of histone H4 acetylated at lysine-5 in chromosomes of *Hordeum vulgare.* The preparations were made by the enzyme maceration squashing method and examined by the confocal laser scanning microscope. Chromosomes at prophase (**A, E**), metaphase (**B, F**) and anaphase (**C, G**) and telophase (**D, H**). **A–D** FITC signals. **E–H** DAPI staining. Bar=10 μm

2. Barley Hordeum vulgare cv. 'Minorimugi'

Barley has four satellite chromosomes and four nucleoli. It had four signals in the metaphase and telophase chromosome complements and eight signals in the prophase and anaphase chromosome complements. Each signal appeared at an NOR. Prophase chromosomes showed more extended signals than the other three stages (Fig. 6) a in the broad bean. Chromosomal regions except the NOR's of barley were weakly labeled by antisera to H4Ac5. At the end of telophase, small dot-like signals appeared throughout the nucleus.

In interphase, five different signal patterns were observed (Fig. 7). In some nuclei, the nucleoli were totally stained (type I). In some nuclei, fiber-like signals emanated from the nucleoli and the surface was brightened. In the case of barley, no prominent knob-like structures resembling those seen in the broad bean (type II) emanated from the nucleoli. Aggregate signals in the nucleus (type III) were observed in some nuclei. Prominent aggregate signals were distributed all over the nuclei except for portions of the nucleoli (type IV) as in the broad bean. String-like or some granule-like signals (type V) could also be seen, as in the broad bean.

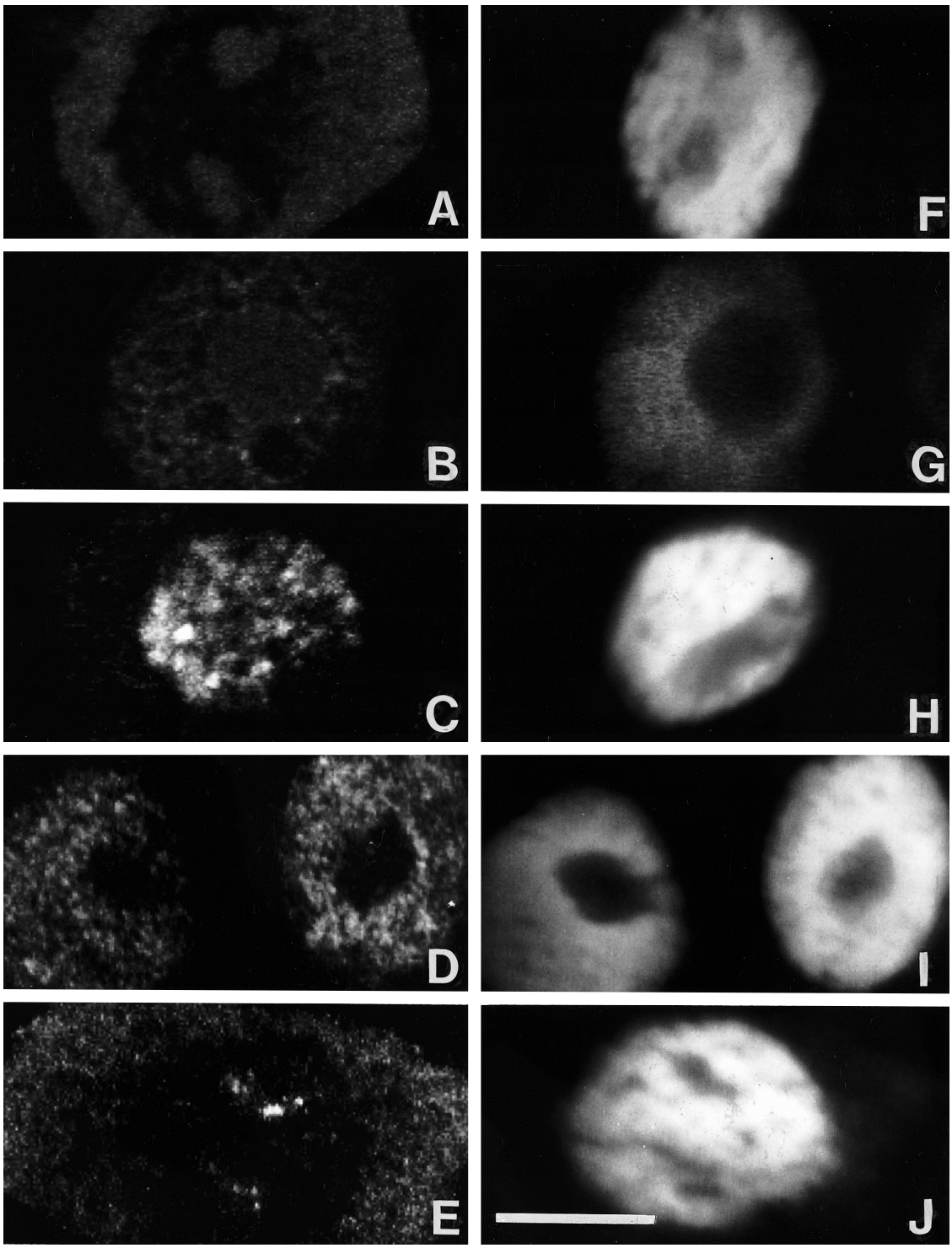

Fig. 7A–J. Labeling with antibodies against histone H4 acetylated at lysine-5 in interphase. **A, F** Nucleolus was fully stained (type I). **B, G** Perinucleolar knob and fibers (type II). **C, H** Aggregate type (type III). **D**, **I** Dot signals distributed through the whole nuclei correlated with the patterns of DAPI (type IV). **E, J** String- or granule-like structures appeared in the nucleoli (type V). Bar=10 μm

Discussion

In the broad bean *Vicia faba* cv. 'Wase soramame' there is a secondary constriction at the interstitial region of the short arm of the longest pair of metacentric chromosomes, forming a large satellite (Evans and Bigger 1961). The secondary constriction is specifically stained by N-banding (Matsui 1974) and silver staining (Schubert et al. 1979). Hizume (1992) reported the results of in situ hybridization with rDNA. On prophase and metaphase, different size signals appeared at both adjacent sides of the secondary constriction of the large metacen-

tric chromosomes. We have shown here that antibodies against histone H4 acetylated at lysine-5 strongly labeled the NORs in mitotic chromosomes of the broad bean. The signals at prophase showed a more extended appearance than those at metaphase (Fig. 1), like the results of silver staining (Hizume et al. 1982). In the case of barley cv. 'Minorimugi', the four NORs were also strongly labeled (Fig. 6). Houben et al. (1996) tried four antibodies directed against H4 acetylated at lysine positions 5, 8, 12 and 16 in metaphase chromosomes of the broad bean. Their results are supported by the present conclusion about the NORs, although they labeled isolated metaphase chromosomes, while the present study used intact cells and observed the signals in interphase nuclei.

The antiserum to histone H4 acetylated at lysine-5 has been used to label mitotic chromosomes in several species, but there have been few reports describing its staining of nuclei, especially in plants. In this research, five different labeling patterns of anti-acetylated histone H4 antibodies were observed in interphase cells in both broad bean and barley. Type I (staining throughout the nucleolus) was observed in the cells that had a condensed nucleus and that were differentiated, such as epidermal cells. This type was considered characteristic of the G_0 phase. Hizume (1992) reported that the interphase nucleus of the broad bean showed two large and two small signals of rRNA genes associated with nucleoli. Rawlins and Shaw (1990) observed the three-dimensional organization of rDNA in interphase nuclei of *Pisum sativum* and found that the tetrahedral internucleolar labeled structures emanating from perinucleolar sites lay in discrete domains, using a cDNA probe to the 18, 5.8 and 25S rDNA sequences. Shaw et al. (1993) observed the same tetrahedral perinucleolar structures in the broad bean, using a sense probe to the rDNA. In those tetrahedral structures, two big and two small perinucleolar knobs were very similar to structures defined by the type II labeling observed in this study. Thus, type II labeling seemed to identify sites of nucleolar transcription. The knob of type II in barley was not as developed as in the broad bean; only the brightness at the nucleolar surface and fiber-like signals were observed. Both cellular capacity and nuclear capacity were large, so it was thought that type II appeared only after S phase – in other words, the G_2 phase.

Cardoso et al. (1993) and Krude (1995) observed that DNA synthesis occurs as a conjugated chromatin domain using anti-BrdU antibodies. The signals of type III showed a similar conjugated appearance possibly reflecting DNA synthesis. Type IV was observed in comparatively small cells and the same dot-like signals appeared in late telophase. This type was considered the G_1 phase. Delgado et al. (1995) reported that the pTa 71 probe, 9 kb EcoRI fragment of the ribosomal DNA from wheat, hybridized to two well-defined sites in peri-nucleolar regions, and fainter labeling showed fine traces of signals connected to the strongest sites and extending into the nucleolus, often joined to less brightly fluorescent traces. In the present research, a similar signal pattern was observed (type IV). The type V signals corresponded to the late-replicating nature of the ribosomal DNA region.

Each signal type could be arranged in the cell cycle as follows. NOR was specifically labeled strongly in the mitotic phase. Many dot-shaped signals appeared at the end of the telophase. The dot-shaped signals appeared in the whole nucleolar area in the G_1 phase. In the next S phase, aggregate signals appeared and the nuclear capacity increased. String-like or granule-like signals appeared at the end of the S phase. Fiber-like or knob-like signals apepared in the G_2 phase. The above order was arrived at by comparing the DAPI images and FITC signals.

Labeling patterns of anti-AcH4 in nucleoli observed in this research were similar to the results of in situ hybridization with rDNA probes (Hizume 1992; Rawlins and Shaw 1990; Delgado et al. 1995), a sense rDNA probe (Shaw et al. 1993) and anti-BrdU antibodies (Cardoso et al. 1993, Krude 1995). They suggest that rDNA is associated with hyper-acetylation of histone H4 throughout the cell cycle.

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