

The nucleus: a highly organized but dynamic structure

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Summary

The nucleus in plants and animals is a highly structured organelle containing several well-defined subregions or suborganelles. These include the nucleolus, interphase chromosome territories and coiled bodies. We have visualized transcription sites in plants at both light- and electron-microscopy level by the incorporation of BrUTP. In the nucleolus many dispersed foci are revealed within the dense fibrillar component, each of which probably corresponds to a single gene copy. In the nucleoplasm there are also many dispersed foci of transcription, but not enough to correspond to one site per transcribed gene. We have shown that in wheat, and probably many other plant species, interphase chromosome territories are organized in a very regular way, with all the chromosomes in the Rab1 configuration, all the centromeres clustered at the nuclear membrane and all the telomeres located at the nuclear membrane on the opposite side of the nucleus. However, despite this regular, polarized structure, there is no sign of polarization of transcription sites, or of any preferred location for them with respect to chromosome territorial boundaries. The nucleus is also highly dynamic. As an example, we have shown by the use of a green fluorescent protein fusion to the spliceosomal protein U2B' that coiled bodies move and coalesce within the nucleus, and may act as transport structures within the nucleus and nucleolus.

Introduction

It is clear that the nucleus is the site of a multitude of complex biochemical processes including DNA replication, gene transcription and transcript processing. It is also clear that the nucleus has a complex, and still poorly understood, ultrastructure. However, progress is now being made in unravelling nuclear structure and in relating nuclear organization to biochemical functions.

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Several types of subnuclear compartment are now known, and others probably remain to be discovered (for recent reviews see Strouboulis & Wolffe, 1996; Lamond & Earnshaw, 1998). The most obvious is the nucleolus – the site of transcription of the ribosomal rDNA and of the biosynthesis of preribosomal particles. The nucleolus in turn is highly organized into distinct functional regions, which in most plant nucleoli can be interpreted as a multilayered organization of the subsequent steps of pre-rRNA maturation and ribosome assembly (Shaw & Jordan, 1995; Brown & Shaw, 1998).

Mitotic chromosomes have been visualized by microscopy for a very long time, but interphase chromatin structure is not easily interpretable. However, there is accumulating evidence that interphase chromosomes occupy distinct regions of the nucleus, often referred to as chromosome territories. This has been demonstrated in mammalian cells by the use of specific chromosome *in situ* 'paints' (Cremer *et al.*, 1988; Lichter *et al.*, 1988). It has been further suggested that the division of the intranuclear space into chromosome territories and intrachromosomal spaces is important in nuclear activity (Cremer *et al.*, 1993; Cremer *et al.*, 1995). Sites of transcription, and thus active genes, have been proposed to be located at the outside of the chromosome territories, so that RNA transcripts are produced near the intrachromosomal spaces, which would provide an efficient route for export from the nucleus (Kurz *et al.*, 1996).

Finally, there are several, as yet poorly understood, intranuclear structures such as speckles and coiled bodies (CBs) (Lamond & Earnshaw, 1998; Matera, 1998). These have been visualized mainly by probes to the components they contain, notably snRNAs and spliceosomal proteins. Coiled bodies, originally identified by Ramon & Cajal (1903), are probably the best characterized of these structures, and are particularly prominent in plant cells (Beven *et al.*, 1995). In addition to spliceosomal components, CBs contain various nucleolar components such as fibrillarin, and the presence of the protein coilin has often

been used as a diagnostic probe for CBs (Lamond & Earnshaw, 1998; Matera, 1998). In this paper we review recent progress in our laboratory in the functional characterization of these various subnuclear compartments in plants.

Materials and methods

Plant material

Seeds of *Pisum sativum* (cv Alaska) were imbibed in aerated water for 12 h then germinated at 18 °C for 2 days on water-soaked tissue paper. Seeds of *Triticum aestivum* (cv Chinese spring/1R disomic addition and Chinese Spring 1A/1R translocation) were germinated on water-soaked filter paper for 3 days. Vibratome sections (TAAB series 1000 vibratome) approximately 30 µm thick were used for both species. For *Pisum sativum*, BrUTP incorporation and fixation and fluorescence *in situ* labelling were carried out as described in Thompson *et al.* (1997); for *Triticum aestivum* the methods were as described in Abranches *et al.* (1998).

Probes

18S, ETS (external transcribed spacer) and NTS (non-transcribed spacer) probes for pea were generated as described previously (Highett *et al.*, 1993; Shaw *et al.*, 1995; Beven *et al.*, 1996). Total genomic probes and probes for centromeres and telomeres were as described in Abranches *et al.* (1998).

Confocal microscopy

Confocal images were collected using a Biorad MRC600, a Biorad MRC1024 or a Leica TCS-SP confocal microscope. Images were viewed, interpreted and made into montages for publication using NIH-Image, a public domain image processing program for the Macintosh written by Wayne Rasband, and available by anonymous ftp from zippy.nimh.nih.gov, and Adobe Photoshop. Final prints were made on a Pictography P3000 printer.

BrUTP incorporation for EM immunogold

For EM immunogold labelling BrUTP incorporation into 30 µm vibratome sections was performed as described previously (Thompson *et al.*, 1997) except for tissue permeabilization, which was done by replacing Triton X-100 by Tween 20. The permeabilized tissue was incubated with the transcription mix for 5 min. After the transcription reaction, the sections were washed in MPB (MPB: 100 mM potassium acetate; 20 mM KCl; 20 mM HEPES; 1 mM MgCl₂; 1 mM ATP; 1% v/v thiodiglycol; 2 mg mL⁻¹ aprotinin; 0.5 mM phenyl-

methylsulfonyl fluoride, pH 7.4 with KOH), fixed in 4% formaldehyde (freshly made from paraformaldehyde) and 0.1% glutaraldehyde in PEM buffer (50 mM PIPES/KOH (pH 6.9); 5 mM EGTA; 5 mM MgSO₄, pH 7.4) for 1 h at room temperature and washed in PBS (140 mM NaCl; 3 mM KCl; 4 mM Na₂HPO₄; 2 mM KH₂PO₄, pH 7.4). Then the sections were dried down on to multiwell slides, coated with glutaraldehyde-activated 3-aminopropyl triethoxy silane (APTES, Sigma, Poole, U.K.) and processed for electron microscopy (see below).

In situ labelling for EM immunogold

For *in situ* labelling the root tips were fixed in 4% formaldehyde in PEM buffer for 1 h at room temperature. After washing twice in PBS for 15 min each, 30 µm sections were cut under water and dried down onto multiwell slides coated with glutaraldehyde-activated APTES. After treating the sections with 2% cellulase (Onozuka R-10, Tokyo, Japan) in PBS for 1 h at room temperature, *in situ* hybridization was carried out overnight at 37 °C, as described previously (Highett *et al.*, 1993) with a digoxigenin-labelled probe to the ETS. Then the sections were washed in 0.1×SSC (150 mM NaCl; 15 mM sodium citrate) for 90 min at 50 °C and processed for electron microscopy (see below).

Specimen processing and post-embedding immunogold labelling

The sections were dehydrated through an ethanol series and infiltrated in LR White resin (Agar Scientific, Stansted, U.K.), containing bezoin methyl ether (0.5%), in a series 1 : 1, 1 : 2, 1 : 3 ethanol : resin, and finally 100% resin overnight at -20 °C. A gelatin capsule filled with resin was inverted over each section, and the resin was polymerized at room temperature overnight with UV light. The polymerized blocks were detached from the slide by cooling in liquid nitrogen. Ultrathin sections were cut using a Leica Ultracut E and collected on 4% pyroxylin on amyl acetate rinsed grids.

The grids carrying ultrathin sections were floated in turn on drops of distilled water, PBS and 5% bovine serum albumin (BSA) in PBS. They were incubated with either mouse anti-BrdU (Boehringer), applied 1/20 in PBS (for BrUTP incorporation), or mouse anti-digoxigenin antibodies, applied 1/5000 in 3% BSA in PBS (for ETS *in situ* hybridization), for 1 h at room temperature. After three washes in PBS, the secondary antibody (anti-mouse 5 nm or 10 nm gold, BioCell) was applied 1/25 in PBS for 1 h at room temperature. Finally, the sections were washed in PBS, rinsed in distilled water and counterstained with 2% (w/v) aqueous uranyl acetate. Observation was carried out with a JEOL 1200 electron microscope.

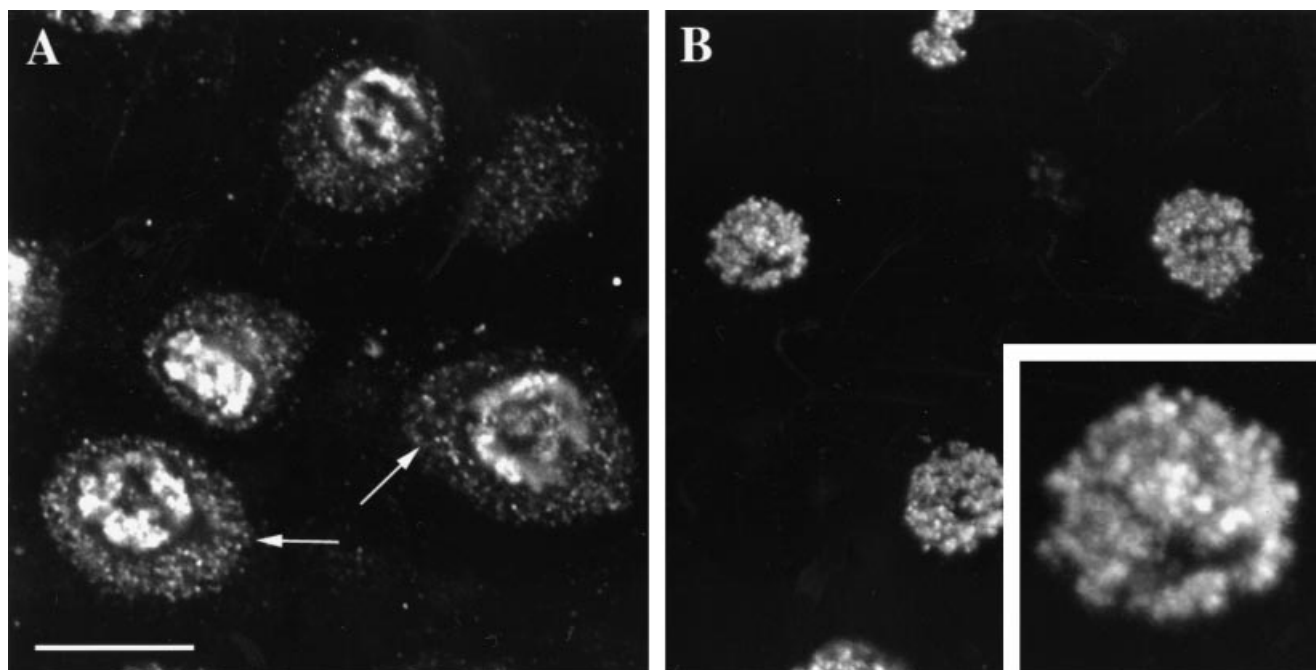


Fig. 1. BrUTP labelling of transcription sites in pea root tissue. Vibratome slices of pea roots, labelled by BrUTP incorporation. (A) Bright labelling is seen in the nucleolus, which comprises many closely packed foci. In the nucleoplasm there are also foci of labelling, but more widely dispersed (arrows). A single confocal optical section is shown. (B) Projection of several optical sections to show labelling in entire nucleoli. Under these conditions only the nucleolar transcription is visualized. Inset: an enlargement of a single nucleolus, showing the closely packed foci within the dense fibrillar component of the nucleolus. Bar = 10 μm .

Generation of U2B''-GFP transformants

The U2B'' constructs generated were described in detail in Boudonck *et al.* (1999). The images shown in this paper resulted from stable, constitutive expression under a $2 \times$ CaMV 35S promoter of a C-terminal U2B''-GFP fusion. The fusion construct was inserted stably into *Arabidopsis* plants and tobacco BY-2 suspension cultures by *Agrobacterium*-mediated transformation (Boudonck *et al.*, 1999). Green fluorescent protein (GFP)-expressing seedlings and cells were imaged using a Leica TCS-SP confocal microscope, with 488 nm excitation. The emission wavelength range detected by the SP system was optimized interactively to give the best imaging of the GFP.

Results and discussion

Transcription occurs at discrete sites in the nucleolus and the nucleoplasm

The results of run-on transcription labelling of pea root tissue at the optical level by BrUTP incorporation are shown in Fig. 1. The strongest labelling is in the nucleolus, where there are many small, bright foci of labelling (estimated at several hundred) distributed through the dense fibrillar component. Under the conditions of our assay, the labelling

in the nucleoplasm is considerably weaker than that in the nucleolus, but still clear and specific. Very gentle permeabilization is necessary to preserve nucleoplasmic transcription; slightly longer permeabilization, as in Fig. 1(b), preserves only the nucleolar transcription sites. In the nucleoplasm, a few hundred discrete foci are seen (arrows in Fig. 1a), distributed fairly uniformly within the volume of the nucleoplasm. Thus, in the nucleolus there are enough foci to correspond to one per gene; in the nucleoplasm there appear to be about an order of magnitude fewer sites than the presumed number of active genes. One explanation for this may be that each nucleoplasmic site represents the transcription of several genes. An alternative is that only the most highly transcribed genes are imaged by this assay, and that many fainter transcription sites are below the detection limit of the technique.

Nucleolar transcription sites probably represent single gene copies

In order to determine the relation between the rDNA and the transcription sites in the nucleolus we combined BrUTP labelling with *in situ* hybridization using different parts of the rDNA repeat as probes (Thompson *et al.*, 1997). Figure 2(a) shows pea root tissue with incorporated BrUTP (red) and *in situ* labelling using the 18S part

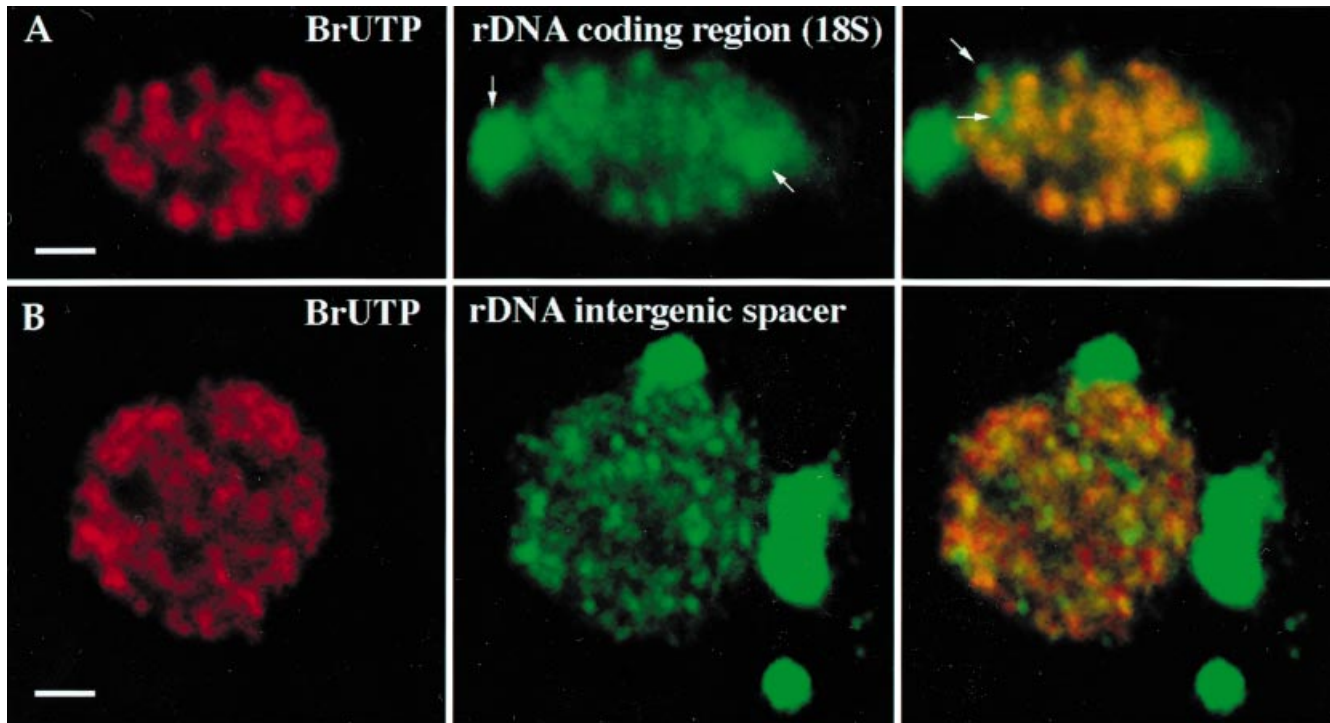


Fig. 2. Comparison of nucleolar transcription sites in pea root tissue labelled by BrUTP with *in situ* labelling of different portions of the rDNA repeats. (A) Single confocal optical section showing double labelling with BrUTP (red) and the 18S part of the transcribed region of the rDNA repeats (green). There are copies of the rDNA that are condensed and not transcriptionally active (arrows), but all the transcription sites labelled by BrUTP correlate with regions of 18S labelling. (B) Single confocal optical section showing double labelling with BrUTP (red) and the intergenic, non-transcribed part of the rDNA repeats (green). There is little overlap of the two probes. Bar = 2 μm .

of the transcribed portion of the rDNA repeat (green). Although there are regions of 18S labelling that do not correspond to BrUTP labelling (arrows), and thus are not transcriptionally active (notably the perinucleolar knobs of condensed rDNA), all the BrUTP labelled transcription sites do correspond to labelling with the 18S probe. By contrast, Fig. 2(b) shows that there is very little correspondence between the non-transcribed NTS regions of the rDNA and the transcription sites. This implies that NTS spacer repeats are not included in the transcription sites, and thus that the transcription sites are likely to represent single gene copies.

Figure 3(a) shows an EM section labelled with gold particles after BrUTP incorporation. Gold particles are seen within the dense fibrillar component of the nucleolus, often clustered together in groups. Around large fibrillar centres, such as the one shown in Fig. 3(a), a regular spacing of 130–180 nm between consecutive clusters is often seen.

Figure 3(b) shows an equivalent section labelled *in situ* with ETS antisense RNA probe. The fine structure is less well preserved than on sections from BrUTP-incorporated tissues, probably because glutaraldehyde fixation could not be used, but the different nucleolar components can be clearly recognized. The labelling is again localized to the dense fibrillar component, giving a pattern of gold distribution

similar to that seen after BrUTP incorporation. Gold particles are clustered and regularly spaced, sometimes around the fibrillar centres (not shown).

Thus, post-embedding immunogold localization of transcription sites, by either detection of BrUTP incorporation sites or ETS *in situ* hybridization, provided similar results. The labelling was located within the dense fibrillar component and the other nucleolar components were unlabelled. Gold particles were mainly seen in clusters, each of which probably corresponds to a single transcription site, in agreement with the results at the optical level. Occasionally these clusters were associated with fibrillar centres, especially the large ones, and a regular spacing of consecutive transcription sites was sometimes observed around them. This regular spacing may be the structural visualization of the molecular organization of rDNA genes in tandem repeats. The association of transcription sites with fibrillar centres suggests that these might serve as anchoring structures, but association of transcription sites with fibrillar centres is not necessary.

Chromosome territories are highly organized

To date it has not been possible to make chromosome paints for any plant species. We have therefore taken an alternative approach to visualizing interphase plant chromosome

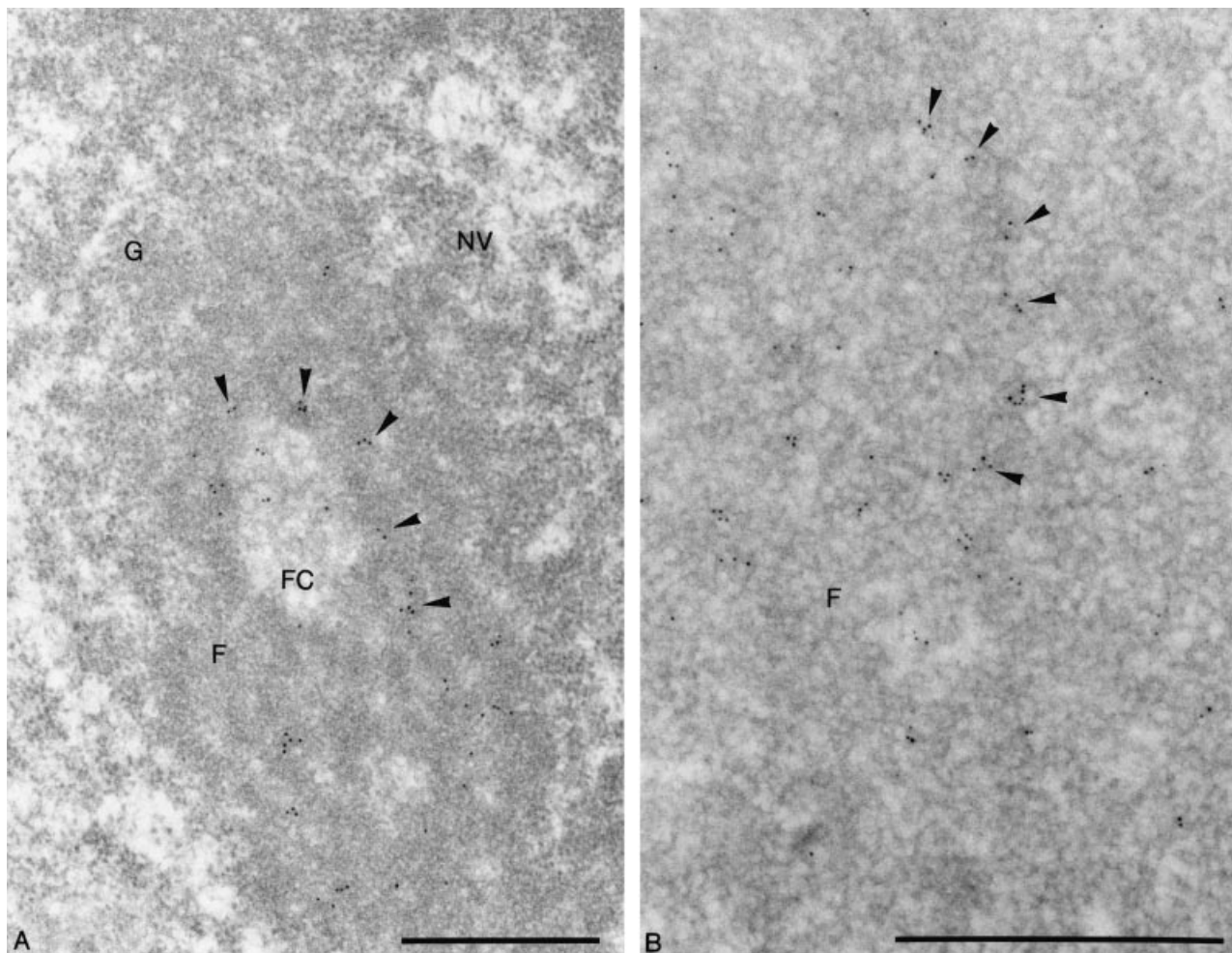


Fig. 3. Post-embedding immunogold EM. (A) Pea root slice labelled by BrUTP incorporation, before fixation and embedding. Incorporated BrUTP was detected by on-section labelling with 10 nm gold. Gold particles are seen over the dense fibrillar component of the nucleolus, often in clusters of three or four gold particles, and sometimes, but not always, in the vicinity of a fibrillar centre. The clusters frequently are regularly spaced (arrows). Bar = 500 nm (B) Pea root tissue labelled by post-section immunogold *in situ* (5 nm), using an antisense probe to the ETS of the rDNA. Again, clusters of gold particles, often regularly spaced, are seen over the dense fibrillar component. Bar = 500 nm. FC – fibrillar centre; F – dense fibrillar component; G – granular component; NV – nuclear vacuole.

territories, involving genomic *in situ* hybridization in wheat (*Triticum aestivum*) lines carrying added or substituted alien chromosomes from other cereal species (Abranches *et al.*, 1998). Figure 4(a) shows root tip cells from a wheat line carrying an additional pair of 1R (rye) chromosomes, hybridized with total genomic rye DNA. The two rye chromosomes lie across each nucleus approximately parallel to each other. The 1R chromosomes have a heterochromatic knob at each telomere, and these can often be clearly seen (arrow in Fig. 4a), showing that the two chromosome arms are lying alongside each other. To confirm this organization we carried out *in situ* hybridization with probes to the centromeres and telomeres (Fig. 4b). This shows that the centromeres lie close together at the nuclear membrane in each nucleus, whereas the telomeres are somewhat more spread out around the opposite side of each nucleus, again located at the nuclear

membrane. These results show that in these nuclei there is a very strong Rabl configuration, with all the chromosomes lying next to each other, and with the two arms of each chromosome lying alongside each other (see diagram in Fig. 4c). Lines of nuclei show a clear common polarity in chromosome orientation, and this polarity alternates in successive nuclei in a line, presumably reflecting the orientation seen at the previous anaphases. It is likely that this chromosome organization extends at least to many other plant species. We have observed it in several cereal species (E. Martinez-Perez, G. Moore, P. J. Shaw, unpublished) and also in the dicot species *Pisum sativum* and *Vicia faba* (Rawlins *et al.*, 1991).

Where are nucleoplasmic transcription sites?

In order to relate the nucleoplasmic transcription sites to

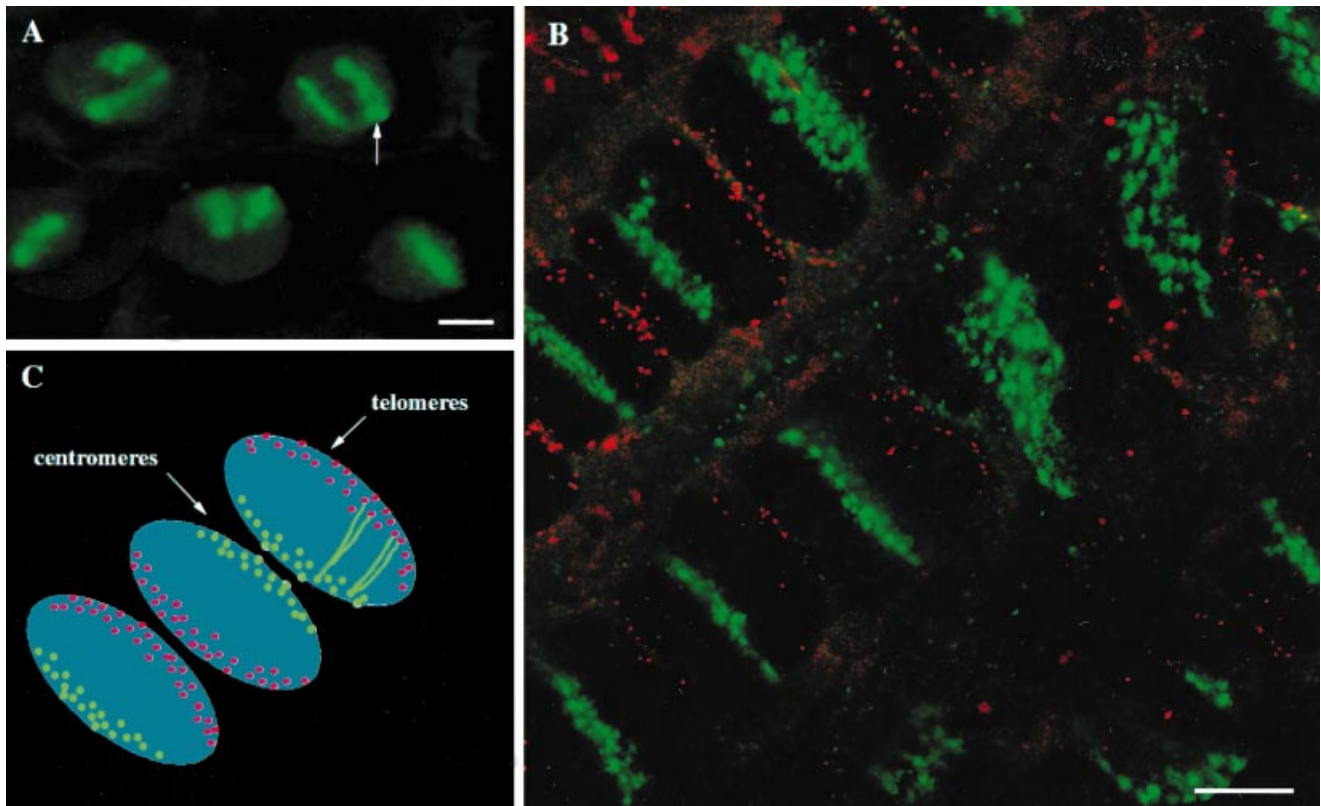


Fig. 4. (A) Wheat 1R addition line labelled by genomic *in situ* hybridization using total rye genomic probe. A single confocal optical section is shown. The two added chromosomes are seen lying across the nucleus, usually parallel to each other. Often the two chromosome arms can be seen lying next to each other in the Rabl configuration (arrow). Bar = 10 μm . (B) Wheat root tissue *in situ* labelled with probes to the centromeres (green) and the telomeres (red). The striking Rabl configuration seen in (A) is confirmed, giving a strong polarity to each nucleus. This polarity alternates along files of cells, reflecting the chromosome configuration in each nucleus at the preceding anaphase. Bar = 10 μm . (C) Diagrammatic representation of the interpretation of the interphase chromosome organization in wheat.

the very regular chromosomal organization in wheat, we carried out double labelling experiments, combining BrUTP visualization of transcription with *in situ* hybridization (Abranches *et al.*, 1998).

Figure 5(a) shows labelling for transcription (red) together with the centromeres (green) in a pair of early G1 wheat root nuclei. The nucleoplasmic transcription sites are fairly evenly distributed throughout the nucleus, with no clear polarity in their density. This is somewhat surprising, because physical mapping of all the wheat chromosomes has shown that the distal (telomeric) portion of the chromosomes is much more gene-rich than the proximal regions nearer to the centromeres (see, e.g. Gill *et al.*, 1996). Although there are other possibilities, the most likely explanation is that there is not a strict relationship between the location of genes on metaphase chromosomes and the physical sites at which they are transcribed during interphase. There is accumulating evidence that active genes are on chromatin loops that can be moved quite large distances in the nucleus depending on their transcriptional state. For example, Brown *et al.* (1997) have shown that a

transcriptional regulator protein called ikaros is localized to domains containing centromeric heterochromatin, and that inactive, but not active, genes are recruited to these domains. Genes or groups of genes being sequestered to specific sites in the nucleus for transcription is consistent with the idea of transcription 'factories' (Jackson, 1995).

Figure 5(b) shows the relation between transcription sites (red) and chromosome territories (green), in this case a single arm translocation of chromosome 1A to 1R (rye). There is no obvious concentration of transcription sites at the periphery of the chromosome territory. In fact, there are some very prominent transcription sites clearly within the interior of the labelled chromosome territory (arrow). Thus, we can find no evidence to support the hypothesis that transcription occurs preferentially at the boundaries of chromosome territories, and suggest instead that transcription sites are distributed throughout the nucleoplasmic region.

Coiled bodies are dynamic subnuclear organelles

We have previously shown that plant nuclei, like animal

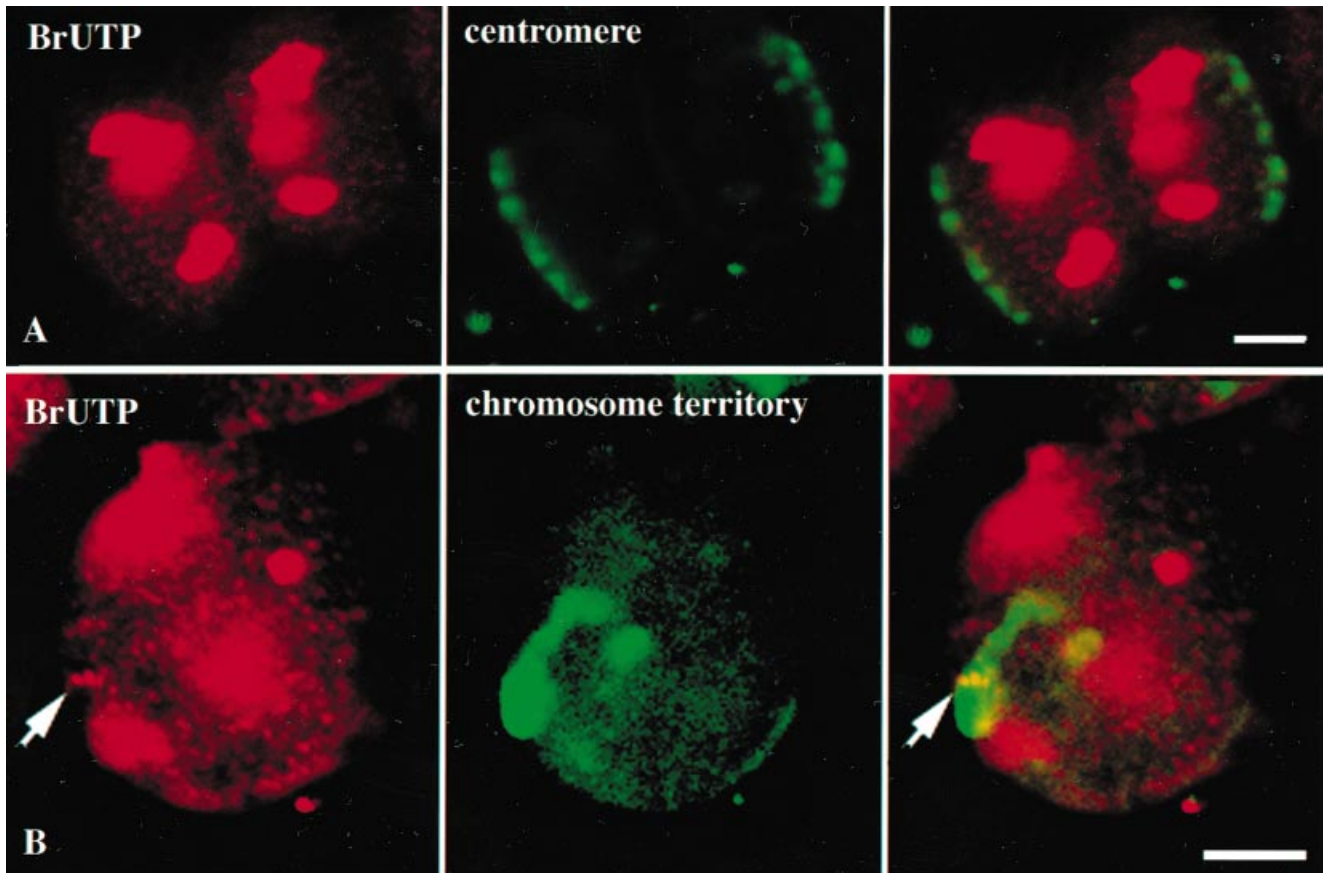


Fig. 5. Location of nucleoplasmic transcription sites in wheat nuclei. A single confocal optical section is shown in each case. (A) Comparison of BrUTP labelling (red) with location of the centromeres (green). The nucleoplasmic transcription sites are not polarized or concentrated towards the telomeric pole of the nuclei, as might be predicted from the physical maps of wheat chromosomes. (B) Comparison of BrUTP labelling (red) with the territory of a single chromosome arm (green). There is no obvious concentration of the transcription sites at the outside of the territory; in fact there are some very bright sites well inside the territory (arrows). Bar = 10 μm .

nuclei, possess coiled bodies which contain spliceosomal proteins, snRNAs and nucleolar components (Beven *et al.*, 1995; Beven *et al.*, 1996). We also showed that in *Arabidopsis* roots, the number of coiled bodies in different nuclei is developmentally determined (Boudonck *et al.*, 1998). As the number of coiled bodies also changes through the cell cycle, the question arises of how these changes in coiled body number occur. To answer such questions we have made a construct containing the spliceosomal protein gene U2B'', which is localized to CBs, fused to a gene for GFP, and expressed this construct in stable transformants of tobacco BY2 suspension culture cells and *Arabidopsis* plants (Boudonck *et al.*, 1999). The fusion protein localizes in the same way as the wild type protein and provides a fluorescent marker for coiled bodies in living cells and plants.

Figure 6 shows a time series from a single BY2 living culture cell expressing U2B''-GFP. A 3-D confocal image stack was collected every hour over a period of 11 h. In each panel of the figure a projection of the 3-D stack is presented in order to show all the CBs present in the

nucleus. Substantial changes in the CBs are apparent over the course of this experiment. Most strikingly, one CB is located at the nuclear periphery until the image at 3 h (arrow). Then it moves to the nucleolus within one hour. Subsequently, this CB moves around the nucleolar periphery, until between 10 and 11 h it coalesces with another CB. Other CB fusions are also probably occurring during the course of the experiment, although not so clearly seen, because approximately six CBs present at the beginning of the period have reduced to three by the end. All of the other CBs visualized undergo smaller movements. In all, we examined 60 cells in this way. Seventy percent of cells (42 cells) showed movements of coiled bodies within the nucleoplasm or within the nucleolus. The large unidirectional movements from the nuclear periphery to the nucleolus shown in Fig. 6 occurred in 25% of these 42 cells. In more than 20% of the 42 cells we observed fusions of two or more CBs. We obtained similar results from time-course analysis of *Arabidopsis* root epidermal cells transformed with U2B''-GFP.

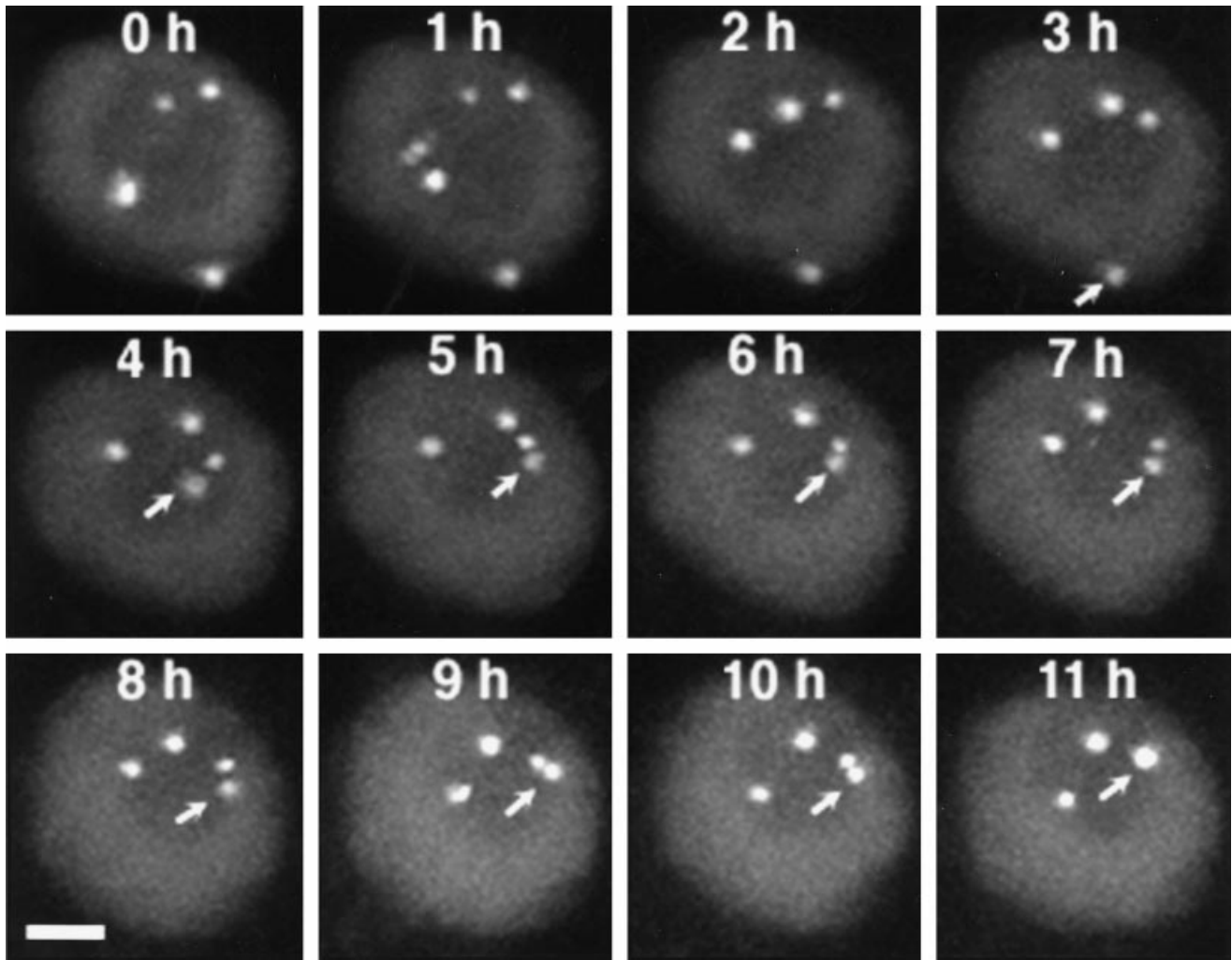


Fig. 6. Time-lapse confocal microscopy of U2B''-GFP in living tobacco BY-2 cells. At each time point a projection of a 3-D confocal stack is shown. The arrowed coiled body (CB) moves from the periphery of the nucleus to the periphery of the nucleolus between 3 h and 4 h, after which it moves along the periphery of the nucleolus and finally fuses with another CB. Other CBs also show movements. Bar = 5 μ m.

The unidirectional movements of CBs we have observed suggest the possibility of a nuclear transport function for CBs. We have previously shown that small nucleolar RNAs (snoRNAs) in plants are transcribed as polycistronic precursor RNAs, which pass through the CBs on their way to the nucleolus where they are required (Beven *et al.*, 1996; Leader *et al.*, 1997). Other studies in animal cells also suggest that CBs may be a sorting or processing structure for nuclear and nucleolar components (Lamond & Earnshaw, 1998). The fusion of CBs we have observed provides a mechanism for the decrease in CB numbers observed during the cell cycle, G1 nuclei having many small CBs and G2 nuclei having fewer, larger CBs. The fact that most CBs undergo small movements most of the time, but that occasionally a single CB within a nucleus undergoes a sudden dramatic movement from the nuclear periphery to the nucleolus, suggests that these large movements may be

triggered by a biochemical signal or modification. It has been shown that changes in phosphorylation can cause CBs to accumulate in the nucleolus (Lyon *et al.*, 1997), and inhibition of transcription by treatment with actinomycin D results in the accumulation of U2B''-GFP in elongated structures around the nucleolus. Thus, there seems to be a link between transcriptional state and snRNP location, which may either be a direct effect or the result of changes nuclear organization or chromatin structure. An interesting question is how do the CBs move through the nucleus – is there an active motor mechanism? Also, can CBs move freely throughout the nucleoplasm or are they constrained to the intrachromosomal channels?

Finally, the production of stable *Arabidopsis* lines expressing U2B''-GFP opens up the possibility of screening for mutants in CB organization or dynamics. In effect, a new range of subcellular phenotypes is available for a

non-destructive mutagenesis screen. In the future this type of approach is likely to be a powerful method for probing subcellular organization and activity by genetic methods.

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