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Review

The architecture of interphase chromosomes and nucleolar transcription sites in plants

Peter J. Shaw,[a,*] Rita Abranches,[a] Ana Paula Santos,[a,b] Alison F. Beven,[a] Eva Stoger,[a,c] Eva Wegel,[a] and Pablo González-Melendi[a,d]

[a] *Department of Cell Biology, The John Innes Centre, Norwich Research Park, Colney Lane, Norwich NR4 7UH, UK*
[b] *Instituto de Tecnologia Quimica e Biologica, Apartado 127, 2781-901 Oeiras, Portugal*
[c] *Institute of Molecular Biotechnology, BiologieVII, RWTH Aachen, Worringerweg 1,52074 Aachen, Germany*
[d] *Centro de Investigaciones Biologicas, CSIC, Velazquez 144, 28006-Madrid, Spain*

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Abstract

Fluorescence in situ hybridization (FISH) coupled with confocal microscopy has been used to reveal the interphase chromosome organization in plants. In wheat and several other related species, we have shown that the interphase chromosomes are in a very well-defined organization, with centromeres and telomeres located at opposite sides of the nuclear envelope—a classic Rabl configuration. In transgenic wheat lines, FISH analysis of metaphase chromosomes has shown that multiple transgene copies can be integrated along a single chromosome, with large regions of intervening genomic sequence. These multiple copies are often colocalized in interphase, suggesting either an ectopic association or a highly reproducible interphase chromatin configuration. Bromouridine (BrU) incorporation has been used to label transcription sites in the nucleolus. Using pea root tissue, we have combined BrU incorporation with preembedding 1-nm gold detection to image the nucleolar transcription sites by electron microscopy. This has revealed many distinct elongated clusters of silver–gold particles. These clusters are 200–300 nm in length and are thicker at one end than the other. We suggest that each cluster corresponds to a single transcribed gene. Serial sectioning of several entire nucleoli has enabled the reconstruction of all the nucleolar transcription sites, and we have estimated that there are 200–300 transcribed genes per nucleolus.

Keywords: 1 nm gold; BrU; 3D microscopy; Chromosome territory; Confocal microscopy; Electron microscopy; Fluorescence in situ hybridization; Genomic in situ hybridization; Interphase chromosomes; Nucleolus; Nucleus; Plant; Rabl configuration; rDNA, rRNA; Serial sectioning; Transcription sites; Transgene; *Triticum aestivum*; *Pisum sativum*

1. Introduction

Much of the fundamental biochemistry of basic nuclear functions such as transcription, RNA processing, and ribosome biogenesis is common to all known eukaryotic organisms. On the other hand there is a huge diversity of large-scale structure between and within the different eukaryotic kingdoms—plants are very different organisms from animals. The comparison of subcellular organization between phylogenetically diverse organisms has the potential to provide powerful insights into the relation between biochemical processes and the associated structures and organization. Thus where organization is similar across phylogeny, this suggests that such organization is necessary for function. Where it differs, this gives clues to the range of viable organization. In this review we shall outline some of our recent results in two areas of subnuclear organization in plants: the structure of interphase chromosomes and the organization of transcription of rRNA genes in the nucleolus.

2. Interphase chromosome organization

Bread wheat (*Triticum aestivum*) has provided a useful plant system for the study of chromosome organization,

* Corresponding author. Fax: +44-1603-450022.
E-mail address: peter.shaw@bbsrc.ac.uk (P.J. Shaw).

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besides being of considerable interest as an important crop species. In common with more than half the known plants, wheat is a polyploid species, maintaining three closely related genomes (A, B, and D), so that most somatic cells are hexaploid ($2n = 6x = 42$). Plant breeders have generated many lines in which extra chromosomes or chromosome arms from other cereal species such as rye or barley have been added or substituted into the wheat genome. Several commercial wheat lines carry such alien chromosomes, which are fully active and have been chosen to confer useful traits. These lines provide powerful tools for cytological analysis, since the alien chromosomes can be specifically labeled by fluorescence in situ hybridization using a total genomic probe for the other species (Schwarzacher et al., 1992). This provides an alternative approach to conventional chromosome paint, which has not so far been successfully applied to plants (Schubert et al., 2001).

Examples are given in Figs. 1a and b, which show images from a line carrying the rye 1R chromosomes in addition to the wheat complement (Abranches et al., 1998). These images are immediately very informative about the interphase chromosome architecture. The labeled chromosomes are seen as elongated structures stretching from one side of the nucleus to the other. In some cases the two chromosome arms can be differentiated as two strands lying next to each other, and the two homologous chromosomes are approximately parallel to each other, although generally separated by intervening chromosomes. The 1R chromosomes carry heterochromatic knobs at the two telomeres, which are easy to locate. This chromosomal arrangement is thus very well defined, with the centromeres located at the nuclear envelope, the chromosome arms folded together, and the telomeres located at the opposite side of the nuclear envelope. This organization was first suggested many years ago by Rabl (1885), who proposed that the anaphase chromosome configuration would persist into the subsequent interphase. In situ labeling with probes for all the centromeres and telomeres confirms that this arrangement is common to all the wheat chromosomes in these root nuclei (Figs. 1c and d). Many such labeling experiments have shown that this strict Rabl configuration is common to all of the cell types so far analyzed in wheat (Aragon-Alcaide et al., 1997, 1998; Abranches et al., 1998), and also extends to many other related cereal species (Martinez-Perez et al., 2000, 2001).

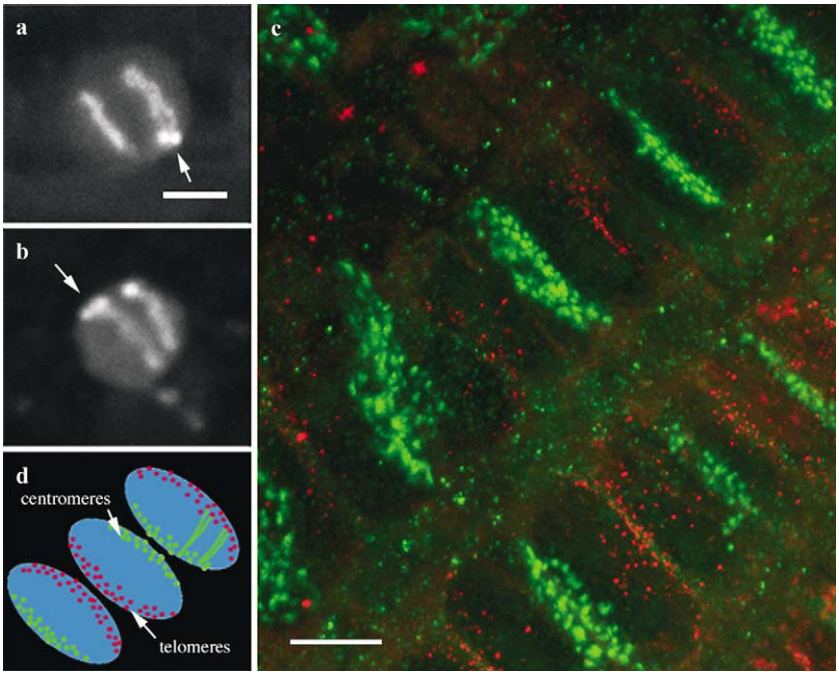

Fig. 1. Chromosome organization in wheat. (a, b) Root cells from wheat 1R addition line, in which a pair of rye chromosomes (1R) is present. The rye chromosomes have been labeled by genomic fluorescence in situ hybridization using a total rye genomic DNA probe, after fixation and vibratome sectioning (see Abranches et al., 1998, for experimental details). Each image is a projection of a confocal series of optical sections. The chromosomes stretch across the nuclei, the two arms next to each other, and the two labeled chromosomes are usually parallel to one another. In the two cells shown, the two arms can be distinguished, and the telomeres can be recognized by the heterochromatic knobs (arrows). (c) Wheat root tissue double-labeled by fluorescence in situ hybridization with probes to the centromeres (green) and telomeres (red). Projection of confocal optical section stack. (d) Diagram showing the organization of the chromosomes as the Rabl configuration. The chromosomes are parallel to one another, with the centromeres clustered on one side of the nuclear periphery, and the telomeres somewhat more dispersed on the other side of the nuclear periphery. A common (alternating) polarity is often maintained through the lines of cells as in this image. Bars, 10 μm.

It is likely that a Rabl chromosome arrangement similar to that we have observed in wheat is shown by many other plant species, especially those with predominantly large, metacentric chromosomes. For example, an arrangement of telomeres consistent with a Rabl organization was shown by *Vicia faba* (bean) and *Pisum sativum* (pea) (Houben et al., 1995; Rawlins et al., 1991). This extends this mode of chromosome organization to dicot species. On the other hand, the chromosome arrangement in *Arabidopsis thaliana*, which has much smaller chromosomes, does not appear to show a Rabl configuration (Fransz et al., 2000; Lysak et al., 2001). Neither, it appears, do sorghum, rice, and maize (Dong and Jiang, 1998). However, these species have so far only been analyzed on squashed preparations. A Rabl organization has been shown for yeast (*Saccharomyces cerevisiae*), with the telomeres often located at the nuclear envelope (Jin et al., 2000). The situation is more complex in animals. In *Drosophila*, many cell types show a Rabl configuration, and in polytene nuclei such as in the salivary gland cells, the centromeres are fused into a single chromocentre (Marshall et al., 1996). Mammalian nuclei do not generally show a Rabl configuration. The interphase chromosome territories show a more complex structure (Dietzel et al., 1998; Cremer and Cremer, 2001; Sadoni et al., 1999), and neither the telomeres nor the centromeres are generally located at the nuclear envelope (e.g., Boyle et al., 2001; Cerda et al., 1999; Croft et al., 1999).

The obvious question is why do some species, or cell types, adopt a Rabl configuration, and others not. Physical constraints may play a role in this. It could be suggested that large chromosomes would be more difficult to move around the nucleus, and would thus be more likely to remain in their anaphase configuration during the subsequent interphase. In plants, the nucleus is probably well protected from exterior physical forces, and its contents may remain relatively undisturbed. However these arguments would suggest that plants such as maize ought to show a Rabl configuration, and it does not. Furthermore, yeast, with a very small genome, does have this configuration. This suggests that specific interactions between the telomeres or centromeres and the nuclear envelope may be important. There is much evidence that this is the case for yeast; it will be interesting to discover whether there are specific interactions anchoring the chromosomes in other species. A more profound question is whether the large-scale organization and position of the chromosomes have implications for function—particularly transcription. There is now good evidence that the conformation of chromatin plays an important part in the regulation of transcription, and so it seems very likely that the higher order organization and arrangement of the chromosomes themselves must be a factor influencing transcription.

3. Transgene organization

There is considerable interest in the introduction of transgenes into crop species such as wheat, and the many transgenic lines being produced provide useful material for studies of chromosome and gene organization (Stoger et al., 1999). We surveyed a selection of transgenic wheat lines, which were produced by particle bombardment, like almost all transgenic cereals so far. In our initial survey we examined 12 lines, which had been transformed with a cassette of marker genes (*gusA, bar*), and determined the chromosomal sites of integration by FISH on metaphase chromosome spreads (Abranches et al., 2000). This showed no apparent preference either for particular chromosomes or for particular parts of the chromosomes. However, an interesting finding was that in several lines, multiple copies of the transgenes were integrated into widely spaced loci along single chromosomes.

We chose two lines for further analysis. Line 6, homozygous, carries 5 transgene copies on each homologue of chromosome 4A. In situ labeling on metaphase chromosomes shows two integration sites on opposite arms of the chromosome, one in a subtelomeric position on the short arm and the other about one-third of the arm length from the telomere of the long arm of the chromosome (Abranches et al., 2000). Line 2, also homozygous, contains more than 10 copies of the transgene at 4 distinct sites along the short arm of chromosome 6B, spanning 30% of the length of the short arm of the chromosome (Abranches et al., 2000). When we analyzed the positions of the transgenes in interphase nuclei of root tissue, we found a strong tendency for the multiple transgenes from each chromosome to be colocalized, even when they were widely separated on the metaphase chromosomes by long intervening stretches of several megabases of genomic sequence (Fig. 2).

This is a surprising and unexpected finding. There are two general types of hypothesis to account for it. First, the transgene loci, which contain multiple copies of identical sequences, could be brought together by an ectopic interaction. This either may be a direct consequence of interactions between the homologous DNA sequences at the different loci or may be mediated by an indirect interaction with specific protein structures or other components. Second, this may be a reflection of the underlying chromosome organization. During the original transformation event producing the cell from which the line developed, the bombarding gold particle may have caused breakage and transgene integration in several DNA loops which were located close to each other, but which originated from widely spaced locations along the length of the metaphase chromosome. Thus at metaphase, the loci are well separated, but during interphase the original, specific chromatin organization is reestablished, bringing the integrated transgenes back

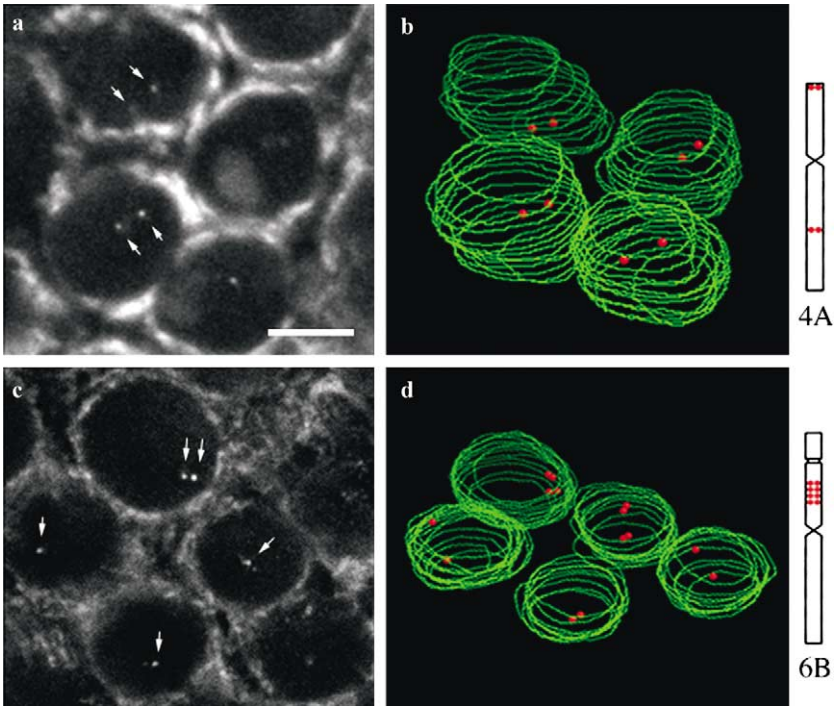

Fig. 2. Transgene organization in wheat root tissue (see Abranches et al., 2000, for experimental details). Two lines are illustrated: line 6 (a,b) which carries 5 transgene copies at 2 sites on metaphase chromosomes (offset from b); and line 2 (c,d), which carries more than 10 transgene copies at 4 sites on metaphase chromosomes (offset from d). Note that in the single confocal sections shown only some of the sites present in the full 3D data from which the models were made are visible. The sites clearly visible on the optical sections shown are indicated by arrows. Image modeling was carried out using Object-Image (an extension to NIH Image written by N.O. Vischer) by drawing manually the limit of the nucleus and marking the localization of the transgene fluorescence sites as dots. NIH image is a public domain program for the Macintosh written by W. Rasband, available via ftp from ftp://zippy.nimh.nih.gov. (a) Single confocal section from line 6 seedling root. (b) Model from complete 3D data stack shown in a. (c) Single confocal section from line 2 seedling root. (d) Model from the complete 3D data stack shown in c. In b each nucleus shows 2 sites—1 per homologue. In d 2 nuclei show 2 sites per homologue, and 3 nuclei show 1 site per homologue. Bar, 10 μm.

together. If this explanation is correct, it implies a highly reproducible interphase organization. We cannot distinguish between these explanations at the moment. The best test would be to analyze the genomic sequences flanking the transgene insertions. If the first explanation is correct, and the colocalization is a consequence of the presence of the introduced sequences, then the genomic flanking sequences will not be colocalized in untransformed wheat plants. On the other hand, if the colocalization reflects a reproducible chromatin organization, then the flanking genomic sequences are likely to be colocalized in untransformed plants.

4. Transcription in the nucleolus

All cells require enormous numbers of ribosomes, and this in turn requires the production of correspondingly large numbers of ribosomal RNAs. In eukaryotic cells, there are many copies of the genes for these rRNAs, which are arranged in the genome as a small number of arrays of tandem repeats. Transcription of the rRNA genes and the subsequent biogenesis of the ribosomal particles take place in the most prominent subnuclear structure, the nucleolus (Hadjiolov, 1985; Huang, 2002; Shaw and Jordan, 1995; Scheer and Hock, 1999). Miller and Beatty (1969) showed many years ago, by an EM spreading technique, a characteristic organization for active rRNA genes, with 50–100 engaged polymerases along the gene and nascent rRNA transcripts of increasing length radiating away from the axis of the DNA—a structure described as a "Christmas tree." However, it has proved very difficult to determine what these transcription units correspond to in vivo. Ultrathin EM sections through nucleoli show various structures, which have been classified as lightly stained fibrillar centers (FC), surrounded by the densely stained regions termed dense fibrillar component (DFC). The remainder of the nucleolus has the appearance of closely packed particles and has been termed the granular component (GC). In plant nuclei, the nucleolus is generally very regular, often close to spherical in structure. It can be very hard to distinguish between the DFC and the GC, and the difference is often one of texture rather than a marked difference of stain density. The DFC generally is a much larger proportion of the nucleolar volume than in animal nucleoli, often accounting for more than 70% of the volume (Shaw and Jordan, 1995).

Many different investigators have used specific probes in a variety of species to localize individual components within the nucleolus—rDNA, rRNA, small nucleolar RNAs, many different proteins. However, although the components recognized by many of these probes must be localized at the transcription sites, they are not necessarily restricted to the transcription sites, and so interpretation of the labeling seen is very difficult. So, for example, one or more rRNA genes must be present at each transcription site, but only a small proportion of the gene copies in the genome are transcriptionally active. Similarly, there must be RNA polymerase I at the transcription sites, but there may be a large proportion of inactive pol I in other places. In one attempt to avoid this problem, we used an RNA probe to detect the externally transcribed spacer (ETS) region of the precursor rRNA as a label in FISH experiments (Shaw et al., 1995). This portion of the transcript is cleaved and degraded very soon after transcription finishes, or possible cotranscriptionally, and so should only be detected at the transcription sites. Typical images of plant nucleoli labeled with ETS showed many small foci closely packed throughout an extended region of the nucleolus, which corresponded broadly to the DFC. Labeling of components involved in later stages of processing of the transcripts, as well as spacer regions excised later in processing, suggested a vectorial model for the arrangement of the initial stages of rRNA processing, with the RNA moving away from the sites of transcription during posttranscriptional processing (Granboulan and Granboulan, 1965; Shaw et al., 1995).

More definitive delineation of the transcription sites has come from direct labeling of the nascent transcripts. Early studies followed the incorporation of tritiated uridine (Granboulan and Granboulan, 1965) but these studies suffered from the lack of resolution inherent in autoradiography. More recently Dundr and Raska (1993) and others (Wansink et al., 1993; Jackson et al., 1993) have introduced bromo-uridine (BrU) as a transcription probe. This analogue is incorporated into nascent transcripts by RNA polymerases and can be detected by antibody labeling. We applied BrU incorporation to pea root tissue and obtained a labeling pattern very similar to that we determined by ETS in situ labeling (Fig. 3)—many small foci closely packed throughout the DFC (Thompson et al., 1997). Recent results using this technique in animal nucleoli reach similar conclusions, showing initial incorporation into nascent transcripts occurring in clusters in the DFC (Koberna et al., 2002). The question then arises of what these foci correspond to—are they individual transcribed genes or clusters of more than one gene?

In order to answer this question we detected the incorporated BrU with 1 nm gold, before embedding tissue in resin for electron microscopy (Gonzalez-Melendi et al., 2001). One nm gold has the advantage of penetrating much better into thick tissue than larger immunogold labels. It requires silver enhancement for detection in EM sections. We cut serial thick sections (0.5 μm), which allowed an entire nucleolus to be imaged in 10–12 serial sections. An example is shown in Fig. 4. The level of labeling was very high—each nucleolus was

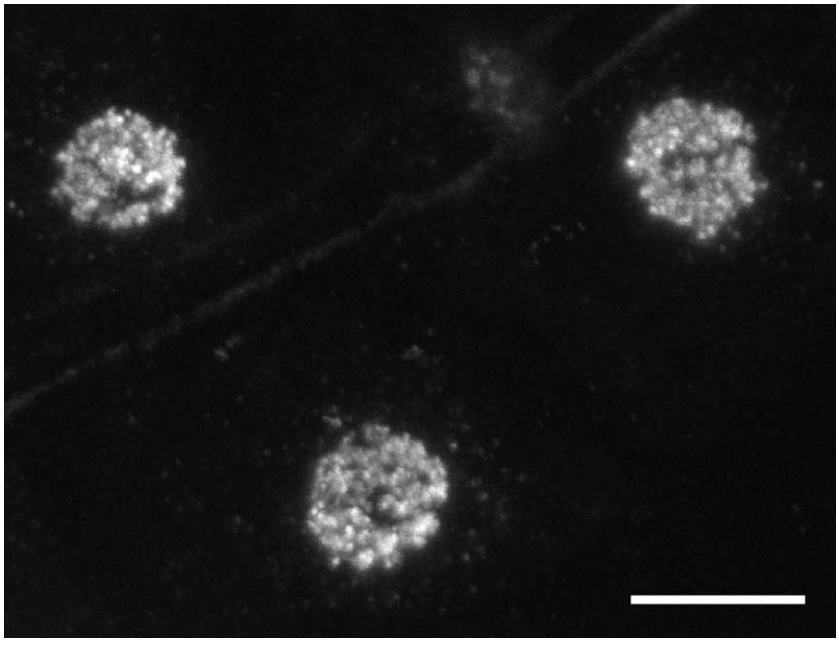

Fig. 3. BrU incorporation into pea root tissue. Pea seedling roots were vibratome-sectioned and allowed to incorporate BrUTP for 2 min before fixing and label detection (see Thompson et al., 1997, for experimental details). A projection of a confocal section stack is shown, including the entire nucleoli. The nucleolar labeling consists of many foci dispersed through the DFC region. Bar, 5 μm.

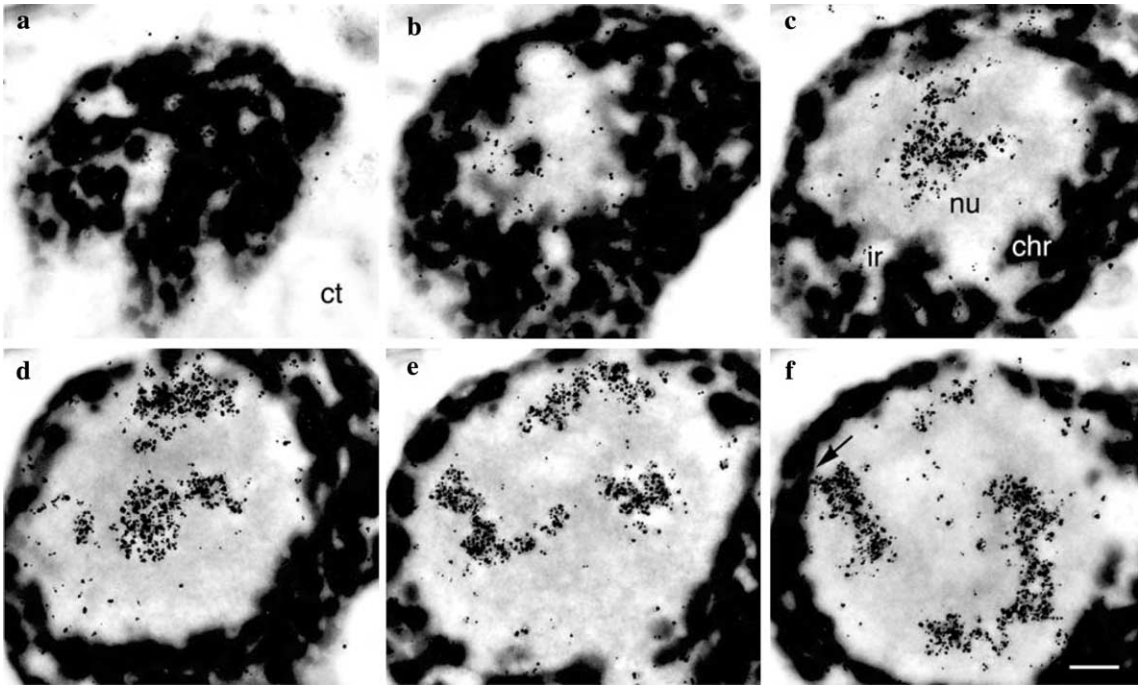

Fig. 4. Detection of nucleolar transcription sites after 5 min incorporation of BrU and preembedding labeling in vibratome sections of pea seedling roots (see Gonzalez-Melendi et al., 2001, for experimental details). Six sections from a complete series of 11 0.5 μm EM sections through an entire nucleolus are shown. The general nuclear morphology is well preserved. Labeling of rDNA transcription sites is seen as dark silver–gold particles within the nucleolar body (nu); there are also some particles in the interchromatin region (ir) at the periphery of the condensed chromatin (chr). Nucleolar transcription sites are localized in specific areas which, on some sections, are seen connected to the extranucleolar condensed chromatin (arrows) that presumably correspond to the knobs containing inactive rDNA genes. Bar, 1 μm.

typically labeled with thousands of silver–gold particles. In many places, clusters of silver–gold particles could be seen, which must correspond to the foci seen at the optical level. Within a single nucleolus, the number of silver–gold particles per cluster was remarkably constant (see Table 1). An example of five such clusters in a single small area of a section is shown in Fig. 5. We propose that each of these clusters corresponds to a single transcribed gene—a compacted Christmas tree. Evidence in favor of this interpretation is first their size and shape; they are elongated and thin (about 300 nm by 50 nm), and they are thicker at one end than the other. Second, the clusters are thicker after longer BrU incorporation times (see Table 1). This is just what would be predicted—longer incorporation would mean that a longer portion of each nascent transcript would be labeled, and thus that the detectable label would spread out further from the axis of the DNA. Finally, because we labeled and imaged entire nucleoli, we were able to count the number of silver–gold particles in each of several

Table 1
Comparison of cluster size after 1 and 5 min BrU incorporation

	1 min BrU	5 min BrU
Mean length (nm)	213.5 ± 43.6	300.0 ± 60.7
Minimum length (nm)	142.9	214.3
Maximum length (nm)	357.1	464.3
Mean width, thin end (nm)	29.7 ± 7.5	30.35 ± 9.4
Mean width, thick end (nm)	54.6 ± 16.7	130.9 ± 43.3
Number of clusters examined	36	30

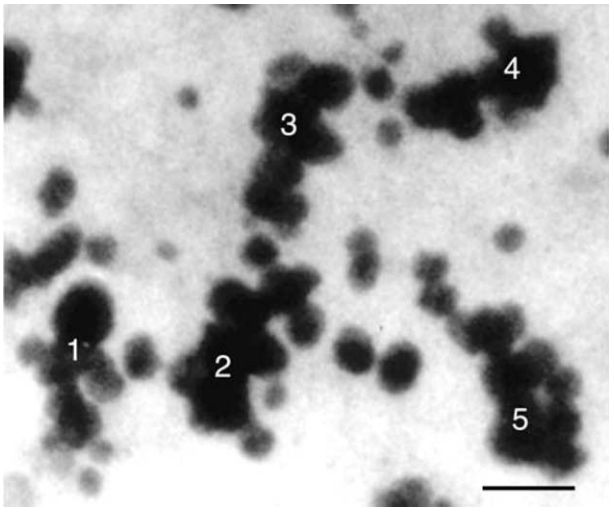

Fig. 5. 3D visualization of individual transcription sites after 5 min BrU incorporation. In many areas of the EM sections, clusters of silver–gold particles were clearly seen. This region shows a region containing a group of five clusters where the orientation was particularly favorable. The shortest cluster is about 200 nm long and 100 nm wide. A gradient of thickness from one end to the other is seen along the clusters. We interpret these clusters as representing individual transcribed rRNA genes, arranged as compacted Christmas trees. Bar, 100 nm.

Table 2
Estimation of the number of nucleolar transcription complexes

	1 min BrU		5 min BrU		
	Nucleolus 1	Nucleolus 2	Nucleolus 3	Nucleolus 4	Nucleolus 5
Number of particles	2708	3540	5032	6328	10 825
Clusters counted	20	25	16	21	17
Particles/cluster	10.7 ± 1.9	12.0 ± 2.3	18.9 ± 2.8	22.7 ± 3.8	36.8 ± 4.8
Clusters/nucleolus	247	294	265	278	293

The number of transcription complexes per nucleolus was taken as the estimated number of silver–gold clusters. Five nucleoli were imaged in entirety from serial sections cut from preembedding labeled tissues after 1 min (nucleoli 1, 2) and 5 min (nucleoli 3, 4, 5) of BrU incorporation. Total number of particles counted on each nucleolus. Single, well-defined clusters were identified and the average number of particles per cluster was calculated for each nucleolus. The number of clusters per nucleolus was estimated by dividing the total number of particles per nucleolus by the average number of particles per cluster, giving an average number of 275.4 ± 17.8 clusters per nucleolus.

nucleoli and use this to estimate the number of active genes. This calculation gave 200–300, in good agreement with other estimates (see Table 2). This is about 5% of the total number of rRNA genes in this species.

The model we have presented for the organization of active rRNA genes in plants is in good agreement with recent results from Koberna et al. (2002), who have shown tight clusters of gold labeling of incorporated BrU within the DFC of animal nucleoli in EM thin sections. They have suggested that these represent sections through the entire transcription units, which are somewhat convoluted, compacted Christmas trees. It also is in agreement with previous results of Melcak et al. (1995) using BrU labeling in isolated onion protoplasts, which showed fibril-like clusters of gold particles. Further support for this organization of transcription units comes from EM thin sections of grasshopper oocyte nucleoli, in which Christmas tree-like structures about 300–400 nm in length have been imaged (Scheer et al., 1997).

There is a long history of controversy about the location of the transcription sites in the nucleolus, with opinions and results divided between the DFC and the FCs. Some of the arguments may have arisen from the labeling of components which may not be restricted to the transcription sites. This is certainly the case with rDNA and RNA polymerase I and associated proteins. Another problem is that the animal models, such as HeLa cell nucleoli, have very condensed DFC regions tightly surrounding FCs, containing several transcription units. The recent results from Koberna et al. (2002) have gone a long way to clarifying this and provide convincing evidence that the transcription units are mostly in the DFC (Huang, 2002). The plant nucleolar organization is much more easily interpreted, since the DFC is more dispersed than in HeLa cells. It is very clear that in plants, the transcription units are widely dispersed through the DFC, and are often well separated from each other. They are also often not close to any visible FC, showing that association with or in an FC is not necessary (Gonzalez-Melendi et al., 2000). Thus the comparison between plant and animal nucleoli illuminates different aspects of the underlying structure organization.

Although there now is good evidence from different species, both plant and animal, that the basic in vivo organization of the transcribed gene is a compacted Christmas tree, many aspects of nucleolar organization remain to be determined, and the clear differences in structure between different cell types, species, and kingdoms need to be explained. What is the higher order organization of the transcription units? Why are they widely dispersed throughout a large DFC region in plants, but restricted to a much smaller DFC adjacent to FCs in animals? Is this different organization related to the large difference in size of the intergenic and spacer regions, which in mammals are much larger than in plants? With complete genome sequences of several higher eukaryotes, including two plants, and with the application of proteomic methods to the nucleolus, we will very soon have a complete inventory of the nucleolar proteins. This will open the way to a much more complete description of the structure of the nucleolus and the relation of its structure to function.

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