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Localization of 5 S genes and transcripts in *Pisum sativum* nuclei

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SUMMARY

We have investigated the position of the 5 S gene clusters in the nuclei of *Pisum sativum* root tip cells, and of their transcripts in the nucleolus, using in situ hybridization and confocal microscopy. Single-stranded RNA probes were produced by in vitro transcription, incorporating fluorescein or digoxygenin label. There are known to be 3 pairs of 5 S gene clusters in this species, which would be expected to give 6 sites of hybridization in G_1 cells and 12 sites in G_2 cells. In presumed G_2 cells, many of the sites appeared as paired spots very close together, which we interpret as the sites on sister chromatids. A clear association of one or more clusters with the nucleolar periphery was observed, and it is possible that this has a functional significance. The transcript labelling within the nucleolus showed a reproducible and highly ordered pattern, consisting of cavities devoid of transcript labelling surrounded by sheets of bright labelling, making a characteristic and often very symmetrical cage-work structure. This labelling pattern may represent an underlying organization of transcript processing within the nucleolus, and is highly reminiscent of the distribution of a previously described nucleolar matrix protein.

Key words: 5 S rRNA, confocal microscopy, gene transcription, 3-D structure, in situ hybridization, nucleolus

INTRODUCTION

Three of the RNA species of eucaryotic ribosomes are transcribed by RNA polymerase I as a single 45 S pre-rRNA precursor within the nucleolus (see Hadjiolov, 1985; Warner, 1989 for reviews). The genes from which they are transcribed occur as a large number of tandem repeats of a single transcription unit, located at a few chromosomal locations - the nucleolar organisers (NORs). The genes for the fourth rRNA species, the 5 S rRNA, are located at different chromosomal locations in almost all eucaryotes, usually on one or more different chromosomes from the 45 S genes, and are transcribed by RNA polymerase III (Hadjiolov, 1985). Even in those few cases where the 5 S genes are within the same repeating unit as the other rRNAs, for example *Saccharomyces* and *Dictyostelium*, the 5 S genes are transcribed by a different polymerase and in the opposite direction (Hadjiolov, 1985). The reason for this separation of the 5 S rRNA genes from the other rRNA genes is unknown, but must have happened early in eucaryotic evolution, and has led to many organisms having more copies of the 5 S genes than of the other rRNA genes. The location of the 5 S genes distant from the NORs implies that they are transcribed in a non-nucleolar location and that the transcripts are then imported into the nucleolus for assembly into pre-ribosomal subunits, but this aspect of 5 S gene organisation and transcription has received little attention.

In most eucaryotes the 5 S genes are clustered into tandemly repeated arrays, some organisms, such as *Drosophila melanogaster* having one or a close pair of clusters (Junakovic, 1980), others having several. A number of plant 5 S genes have been characterised: for example, flax has 5 S genes organised both in long tandem repeats and dispersed throughout the genome (Schneeberger et al. 1989), whereas in *Phaseolus coccineus* there are three sites on two chromosomes (Durante et al., 1977). *Pisum sativum* has about 5,000 copies of the 5 S rDNA repeats per diploid genome arranged as three pairs of tandem arrays, each containing 300-700 kbp of the ~300 bp repeat (Ellis et al., 1988). In situ hybridization on metaphase chromosome squashes has shown that they are located at one interstitial and two sub-telomeric sites on three chromosomes, none of which carry NORs in the standard karyotype (Simpson et al., 1990). The interstitial site shows a stronger signal than the telomeric sites, suggesting that this cluster contains more copies of the genes. In this paper we describe the location of 5 S rRNA genes and transcripts in interphase cells of root tissue of *P. sativum*, using in situ hybridisation with sense or anti-sense single-stranded RNA probes (riboprobes), coupled with 3-D confocal microscopy.

MATERIALS AND METHODS

Specimen preparation

Seeds of *Pisum sativum* L. (cultivar Greenshaft JI430) were imbibed in aerated water for 12 h then germinated at 18°C for 2 d on water-soaked paper towels. The terminal 3-5 mm of the radicle was excised under water, and fixed in 4% (w/v) formalde-

hyde/0.1% (v/v) glutaraldehyde in buffer (50 mM PIPES/KOH (pH 6.9), 5 mM EGTA, 5 mM $MgSO_4$) for 1 h at room temperature. After washing 3 times in TBS (TBS: 25 mM Tris-HCl (pH 7.4), 140 mM NaCl, 3 mM KCl), 30-40 μm vibratome sections were cut under water and dried down on to glutaraldehyde-activated γ-aminopropyl triethoxy silane-coated multiwell slides. Slides were then stored at −20°C or used immediately. Sections of this thickness typically contained 2-3 layers of cells; the great majority of nuclei were entirely contained within the section, and only nuclei that were clearly intact were analysed. Tissue sections were treated four times for 15 min at room temperature with freshly prepared sodium borohydride solutions (1 mg/ml in PBS) (PBS: 140 mM NaCl, 3 mM KCl, 4 mM Na_2HPO_4, 2mM KH_2PO_4, pH 8.0) to reduce autofluorescence caused by glutaraldehyde. Sections were permeabilized with 2% (w/v) cellulase (Onozuka R-10) in TBS for 1 h at room temperature.

Preparation of probes

A single 279 bp repeat from *P. sativum* p5Ss (5 S rDNA clone kindly donated by Dr N Ellis; Ellis et al., 1988) was sub-cloned into the Bluescript SK^+ vector (Stratagene) at the *Bam*HI site. Single-stranded RNA probes were generated as 'run-around' transcripts from the T7 or T3 promoters. The insert was oriented such that T7 polymerase produced sense RNA (complementary to the transcribed strand of the DNA), and T3 anti-sense. Label was incorporated into the probes by including either digoxygenin-11-UTP (Boehringer) or fluorescein-12-UTP (Boehringer) in the in vitro transcription reaction. The probe size was reduced to approximately 200 bases by a mild carbonate hydrolysis (Cox et al., 1984) to improve penetration into the specimen. In order to confirm its specificity the probe was hybridised to blots of restriction digests of the Bluescript vector containing the insert. Since there was also slight labelling of the plasmid band, we used an excess of unlabelled RNA transcribed from the plasmid alone or an unrelated insert to block non-specific binding in the in situ experiments.

Nucleic acid blotting

Digests were run out on 1.2% (w/v) agarose gels using standard conditions (Sambrook et al., 1989), and transferred to Hybond-N filters (Amersham) for filter hybridization experiments. After hybridization with digoxygenin-labelled probe and washing, the probe was detected with alkaline phosphatase-conjugated anti-digoxygenin (Boehringer), developed with nitro blue tetrazolium chloride and 5-bromo, 4-chloro, 3-indolyl phosphate (NBT/BCIP - Promega), used according to the manufacturer's instructions.

In situ hybridization

In situ hybridization to tissue sections was carried out as described previously (Highett et al., 1993). In some experiments single-stranded DNA target was produced by heat-denaturing the tissue sections prior to hybridization, in others a pronase digestion was used (0.1 mg/ml pronase in 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 30 min at 37°C), in others no pre-treatment was carried out. In the cases where fluorescein-labelled probe was used, no further reactions were used for probe detection. Digoxygenin-labelled probe was detected by fluorescent antibody labelling as described previously (Highett et al., 1993). In some control experiments the in situ labelling was preceded by either DNase or RNase digestion as described previously (Highett et al,1993). In some experiments the sections were counterstained with 40 μM 7AAD (7 amino, actinomycin D) for 10 min.

Microscopy

Confocal optical sections stacks were collected using a Bio-Rad Lasersharp MRC-500 confocal scanning head mounted on a Zeiss Universal microscope as described previously (Rawlins and Shaw, 1990). Image processing and interpretation was performed on a Titan super-workstation (Kubota Pacific Computer Company). Image deconvolution was carried out with the 3-D iterative constrained Jannson-van Cittert method using the program DECON3D written by Dr David Agard, and described in Agard et al. (1989), implemented on the Titan computer. Measured point spread functions for the relevant detector pinhole size were used for the deconvolutions (Shaw and Rawlins, 1991a,b). Montages of images were produced for display and interpretation of the various data sets. Alternatively, series of projections were calculated at different angles (Agard et al., 1989) and displayed as stereo pairs or sequentially to give the impression of rotation. In the calculation of projections, either the mean or the maximum intensity along each site line was used, usually the latter. Images were photographed directly from the monitor.

RESULTS

Characterisation of in situ labelling targets

Fig. 1 shows a low magnification picture of a portion of a root section labelled with anti-sense 5 S probe, and counterstained with 7 amino actinomycin D (7AAD) to show the nuclear chromatin. The majority of the nuclei are intact and well-preserved, although two nuclei through which the plane of section has passed are clearly identifiable (arrows). In order to be sure of the targets of in situ labelling we carried out a series of control experiments. These experiments are summarised in Fig. 2. After heat denaturation, the sense probe labelled between six and twelve bright nucleoplasmic spots in each cell (Fig. 2A), but in the absence of heat denaturation no labelling was seen. Thus denaturation was absolutely necessary to produce single-stranded DNA target. After complete digestion of the DNA by RNase-free DNase, as judged by lack of staining with DAPI, no labelling was seen with the sense probe (Fig. 2B), whereas pre-digestion with DNase-free RNase had no effect on the labelling pattern (Fig. 2C). Labelling with anti-sense probe after heat denaturation showed both 5 S genes in the nucleoplasm and 5 S transcripts in the nucleolus (Fig. 2D). Pre-digestion with DNase removed the labelling of the genes (Fig. 2E) whereas pre-digestion with RNase removed the transcript labelling (Fig. 2F). In the absence of heat denaturation, the anti-sense probe labelled only the transcripts within the nucleolus (Fig. 2G), and the labelling was unaffected by DNase (Fig. 2H), but abolished by RNase (Fig. 2I). In other control experiments, labelled transcript from plasmid containing no insert or unlabelled 5 S probes were used, and showed no signal. Thus, depending on the conditions, we were able to label specifically either genes, transcripts or both. Pronase predigestion used in some experiments did not alter the distribution of labelling seen. Therefore pronase digestion was not used in the most detailed reconstructions shown here.

Arrangement of 5 S gene clusters

We analyzed the position of the 5 S gene clusters from confocal data stacks of specimens labelled after heat denaturation either with sense probe (see Fig. 3) to reveal solely the genes, or with anti-sense probe (see Figs 4 and 5) to show both genes and transcripts in the nucleolus. In many

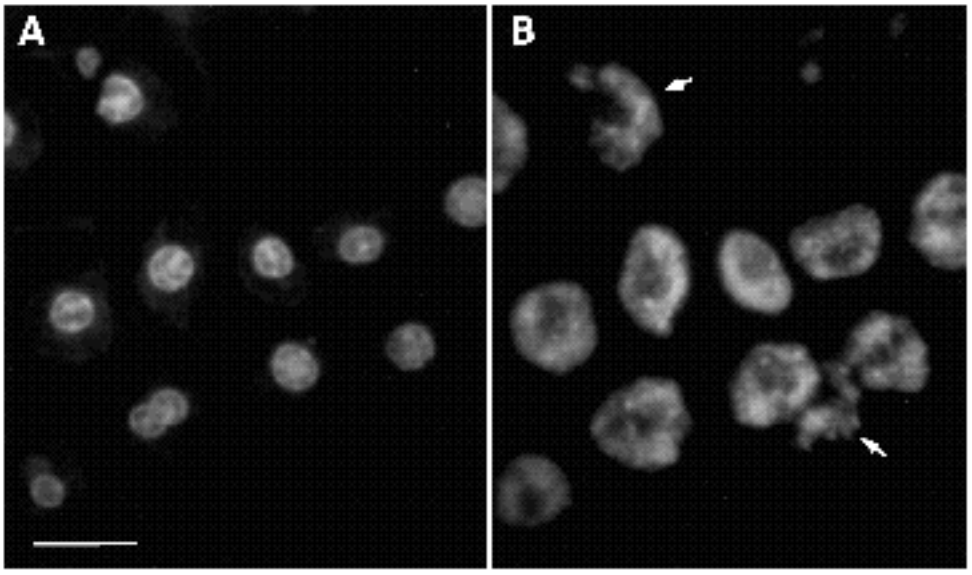

Fig. 1. Low magnification micrograph showing part of a tissue slice labelled with anti-sense 5 S probe (A), and counterstained with the DNA dye 7AAD (B), to show the nuclei. Most of the nuclei are intact and undamaged, but two nuclei are at the section surface and have been sliced through (arrows). A 3-D confocal data stack, consisting of 24 optical sections spaced 1 μm apart has been projected. Bar, 20 μm.

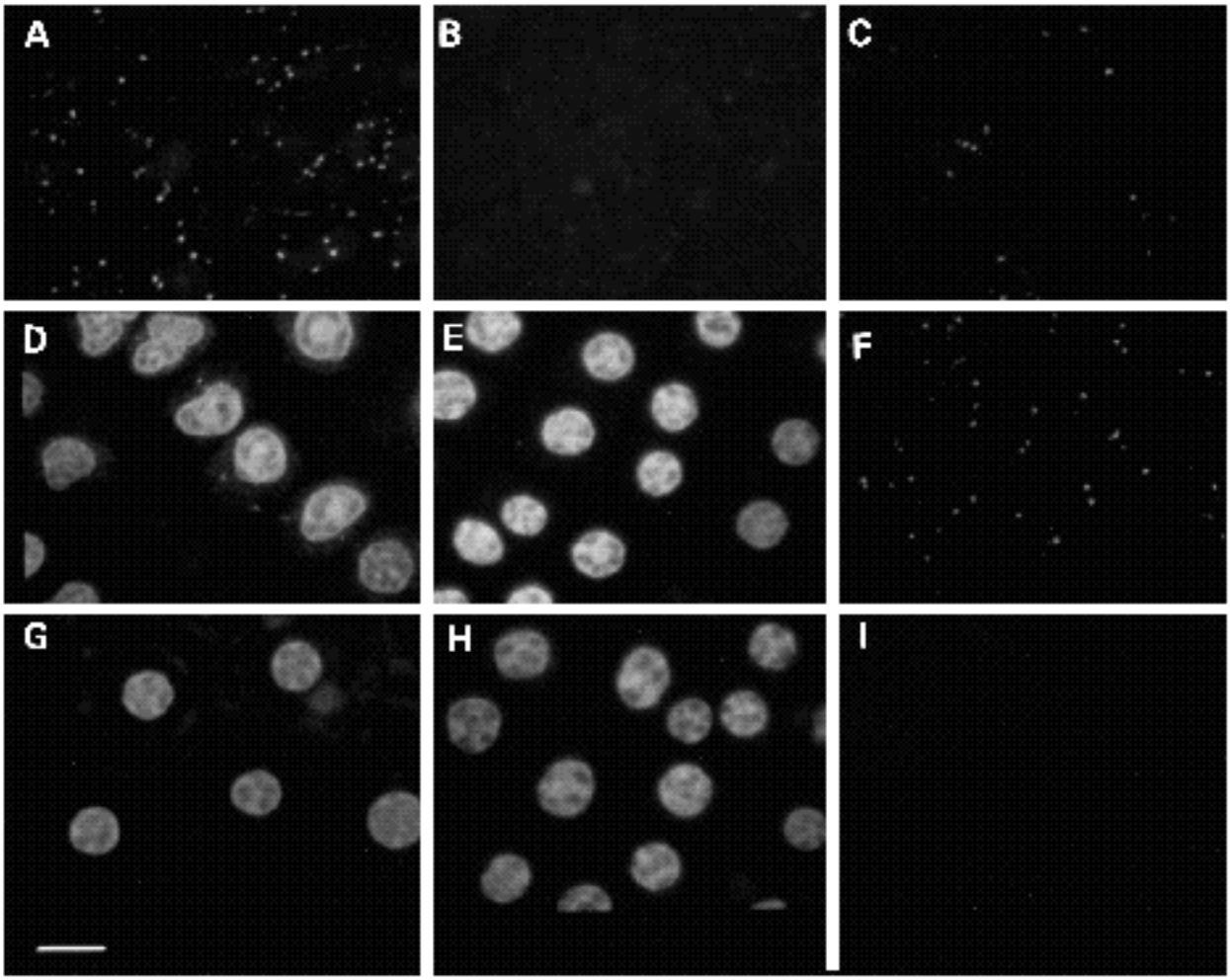

Fig. 2. Nuclease predigestion control experiments. (A) Sense probe, tissue heat denatured but otherwise untreated. Only the gene clusters are labelled. (B) Labelling as in (A), but predigested with DNase. No labelling is seen. (C) Labelling as in (A), but predigested with RNase. The labelling is unaffected. (D) Anti-sense probe, tissue heat denatured but otherwise untreated. Both genes and transcripts in the nucleolus are labelled. (E) Labelling as in (D), but predigested with DNase. The labelling of the genes is abolished, that of the transcripts in the nucleolus remains. (F) Labelling as in (D), but predigested with RNase. The labelling of the genes remains, that of the transcripts is abolished. (G) Anti-sense probe, tissue untreated (no heat denaturation). Only the transcripts in the nucleolus are labelled. (H) Labelling as in (G), but predigested with DNase. The labelling is unaffected. (I) Labelling as in (G), but predigested with RNase. All labelling is abolished. Bar, 5 μm.

Fig. 3. Stereo projection of a single nucleus, labelled with sense probe after heat denaturation. There can be seen 7 nuclear sites of 5 S gene clusters, of which 4 are close pairs (arrowheads). The other 3 sites do not appear as pairs, but we presume one of them at least must represent a pair to give the G_2 complement of 12 5 S clusters. Bar, 2 μm.

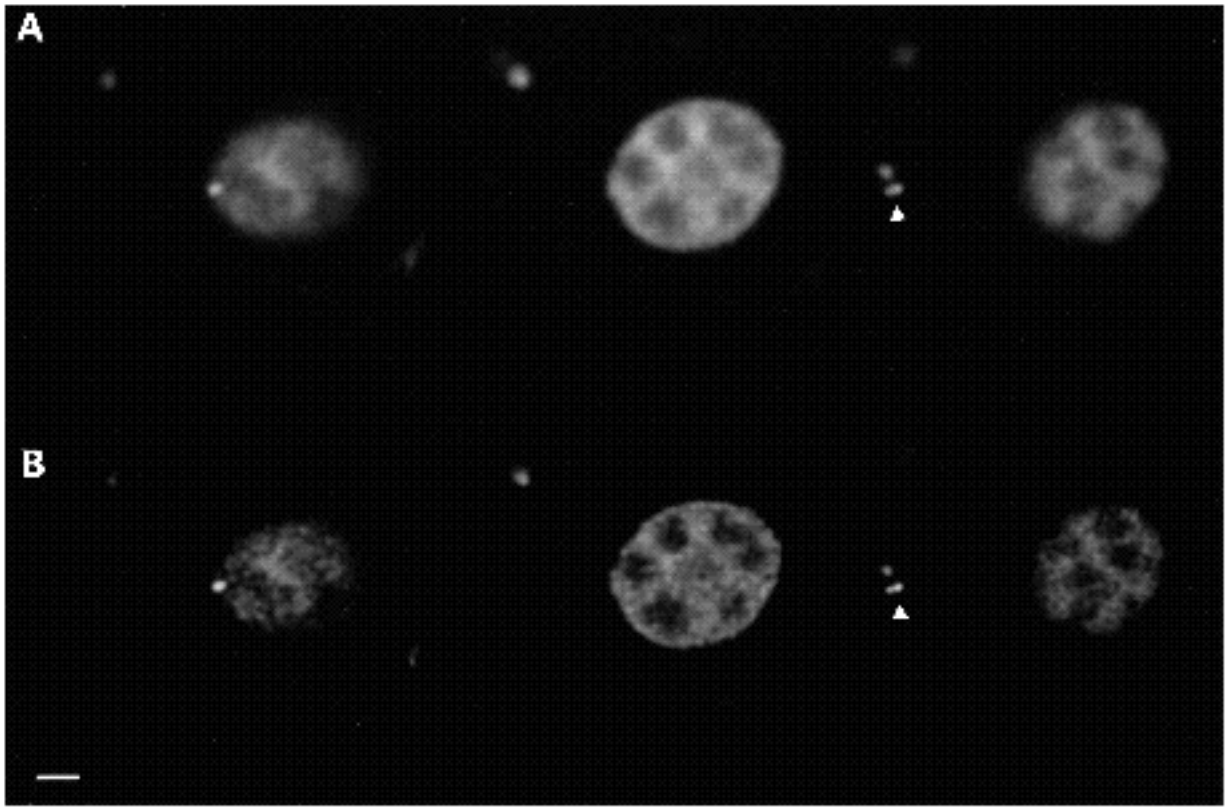

Fig. 4. Three selected confocal optical sections taken from the full 3-D data stack of a single nucleolus labelled with anti-sense probe after heat denaturation. (A) Raw confocal data. (B) The same sections after deconvolution. The nucleolar substructure is much clearer. Also note the clearer resolution of the pair of 5 S clusters (arrowheads). Bar, 2 μm.

cases we used image deconvolution to improve the clarity of the 3-D images, and an example of three optical sections before and after deconvolution is shown in Fig. 4. The improved resolution of the nucleolar substructure is apparent, and the arrowed gene cluster is resolved much more clearly into two sites (the cell is evidently in G_2 - see Fig. 5). We have described the deconvolution procedures in detail previously (Shaw and Rawlins, 1991a; Highett et al., 1993). In most cases the 5 S gene clusters were approximately spherical spots, but in a few cases there was the appearance of a 'tail' emanating from a spot. We examined in detail 20 nuclei chosen at random, including both cells from the actively dividing meristematic regions (10 cells), and other regions (10 cells, including root cap, epidermal and cortical cells). In some cells six bright nuclear spots were seen, suggesting the cells were in G_1 (7 cells from various cell types), in others there were up to twelve spots, from which we assumed that the cells were in G_2 (the

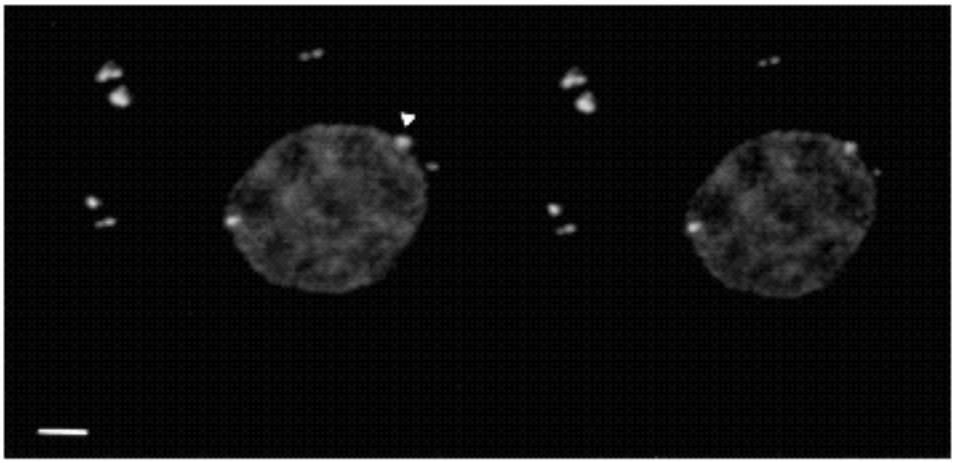

Fig. 5. Stereo projection of the entire data set (after deconvolution) from which the sections shown in Fig. 3 were taken. Four 5 S cluster pairs are clearly seen, and two clusters are associated with the nucleolar periphery. (One of these clusters may be a fifth pair - arrowhead.) Bar, 2 μm.

remaining 13 cells), or at least later in S than the time of replication of the 5 S genes. Often, several of the spots occurred in closely associated pairs as in Fig. 5, which we assume represent gene clusters on sister chromatids. However, in no case were all the spots in an assumed G_2 nucleus paired - generally more than half were paired, and the others apparently single. However the fact that we were not always able to count 12 spots suggests that in some cases the genes on sister chromatids remained too close together to resolve. For example in Fig. 3 there are at first sight 7 spots, of which at least 4 can be resolved into pairs (arrows), making a total of 11 clusters. We assume that one of the remaining spots is an unresolved pair, but this must leave at least 2 clusters on sister chromatids widely separated in the nucleus. Similarly in Fig. 5 there are 4 pairs (arrows) and 4 widely separated single sites, making the G_2 complement of 12.

A striking observation was an association of one or more clusters with the nucleolar periphery. Of the twenty cells examined, only one apparently lacked any nucleolar-associated cluster (and the nucleolus in this cell was much the smallest, and presumably least active). In seven cells there were three nucleolar-associated clusters and in six cells there were two associated clusters. In order to test the statistical significance of this observation we counted the total number of clusters in each nucleus, and the number of these that were associated with the nucleolus. We also estimated the approximate nucleolar volume and total nuclear volume of each cell by measuring their respective radii. A cluster was classified as nucleolar associated if its intensity was continuous with the nucleolar periphery. Given the size of the spots and the optical resolution limit, this would mean within approximately 0.5 μm of the nucleolus. We therefore calculated the volume of a shell 0.5 μm wide around the nucleolus and expressed this as a fraction of the nuclear volume excluding the nucleolus. From this data we calculated the probability that the observed number of clusters would occur in this volume by chance. That is, the null hypothesis was that the clusters were randomly distributed throughout the accessible nuclear volume (the total nuclear volume excluding the nucleolus). These individual probabilities for each nucleus were combined by calculating the sum of $-2.0*\log_e P$, which has been shown (Fisher, 1934; Snedecor, 1965) to be distributed as chi-squared with $2N$ degrees of freedom for N probabilities, where P is the previously calculated probability for each nucleus of obtaining the observed distribution by chance. This sum gave 65.06, which shows a combined probability of less than 1% for 40 degrees of freedom. Thus we may reject the null hypothesis with 99% confidence, and conclude that there is a significant association of 5 S genes with the nucleolus.

5 S transcript labelling in the nucleolus

Labelling with anti-sense probe, either with or without heat denaturation, showed a very clear nucleolar distribution (see Figs 4, 5 and 6). The intensity of labelling in the absence of heat denaturation was increased by pre-digestion with pronase, which would be expected partly to remove protein from ribonucleoprotein complexes and make the RNA more accessible to the probe, but the distribution of the labelling was the same whether pronase digestion was used or not. The nucleolar labelling pattern is highly reproducible and characteristic, with bright sheets of label surrounding dark voids or chambers. In small, and presumably less active, nucleoli there is often a single central void, surrounded by a brightly labelled peripheral shell. In larger nucleoli, there are several internal chambers surrounded by sheets of label that extend through the nucleolus as well as around the periphery. In optical sections near the centre of a nucleolus this gives a characteristic 'cart-wheel' appearance. In many of the largest nucleoli, there is an inner shell surrounding a central void, with other voids between this and the outer shell, usually forming a symmetrical structure. The larger the nucleolus, the more chambers are visible. In very early G_1 cells, there may be up to 4 smaller nucleoli present (Rawlins and Shaw, 1990), which then fuse into a single large nucleolus. An example of a nucleus, which we presume to be in early G_1, with 3 nucleoli is shown in Fig. 7. The nucleoli in these cells show a similar pattern, although they have fewer chambers (in fact, the smallest nucleolus in Fig. 7 has only a single chamber with a surrounding rim of labelling). We have seen a few cells where two or more nucleoli are apparently in the process of fusing; in these cases the fusion seems to begin by merging of the outer rims of labelling. The other nucleus in Fig. 7, which we presume to be in G_1, is typical of the pattern seen in

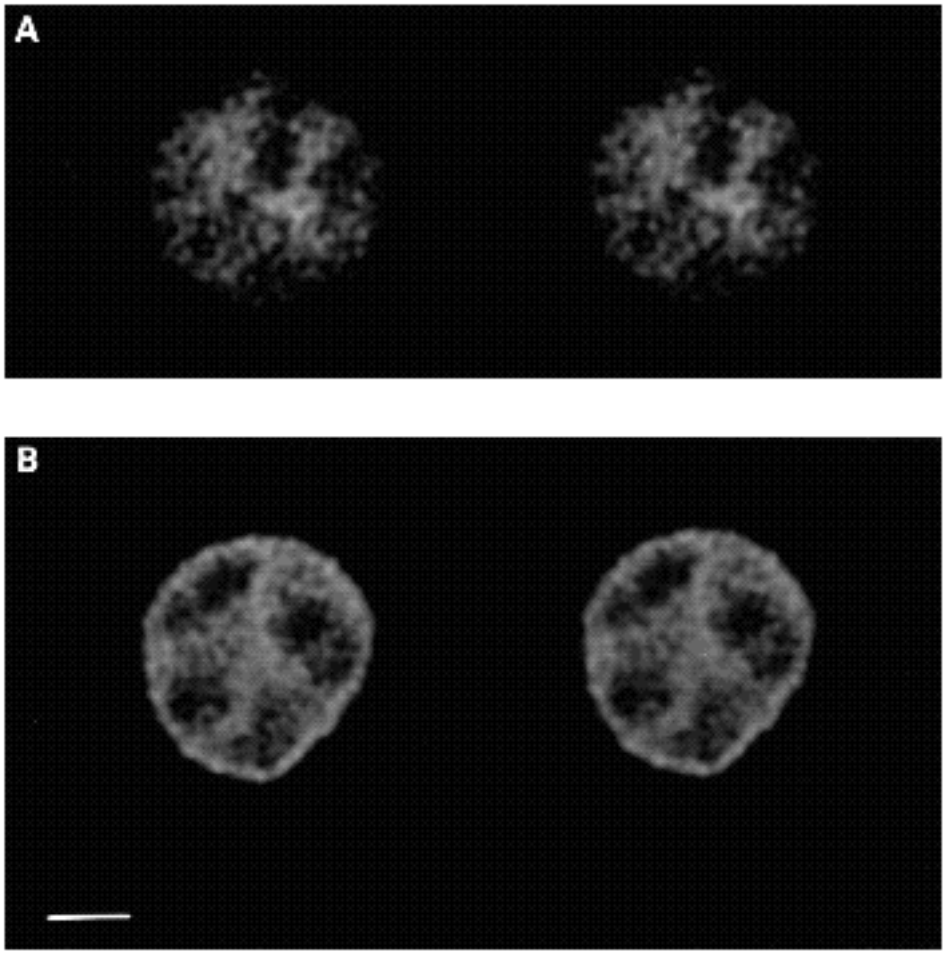

Fig. 6. Stereo projections from limited slices (approximately 1 μm thick - 5 optical sections) from two nucleoli, labelled with anti-sense probe, without heat denaturation. (A) Slice at the top of the nucleolus, showing the fibrous substructure. (B) Slice near to the centre of the nucleolus, showing the cavities within the nucleolus devoid of 5 S transcript. Bar, 2 μm.

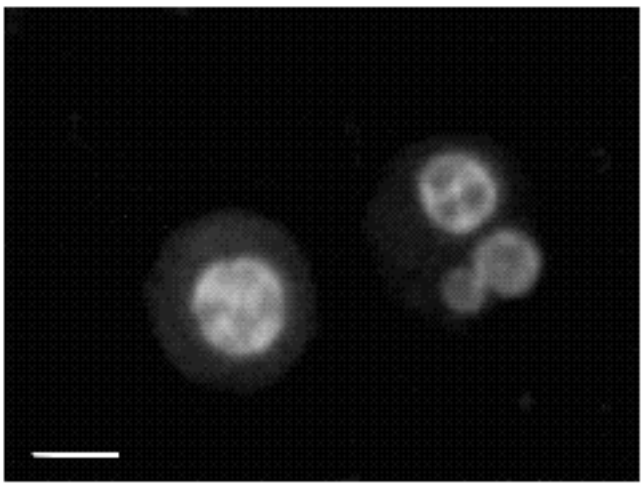

Fig. 7. Two nuclei labelled with anti-sense probe, one with a typical small single nucleolus, one with 3 small nucleoli, both presumed to be in early G_1. Similar substructure is visible in the small multiple nucleoli, although with fewer chambers. The smallest nucleolus seems to contain a single chamber. Here 14 confocal optical sections through the nuclei have been projected. Bar, 5 μm.

small nucleoli, with no central void and fewer chambers. There is considerable substructure visible in the labelling, and this is particularly clear after image deconvolution (see Fig. 5). The labelling comprises strands or fibres and is very obvious in optical sections and projections at the top of a nucleolus (see Fig. 6A), which constitute glancing sections through the nucleolar periphery.

DISCUSSION

Judged by the number of 5 S clusters observed, some of the cells we have analyzed are in G_1, some in G_2, as we would expect. In all the G_2 cells, some of the 5 S clusters are seen as closely associated pairs of spots. The obvious interpretation would be that these represent the 5 S genes on sister chromatids and that at these positions the chromatids are separated by a little more than the optical resolution limit - i.e. about 0.25 μm. However in all those nuclei containing such pairs there are other sites that do not seem to be pairs. Although in some cases this may indicate that the chromatids are separated by less than the optical resolution limit and so appear as single sites, in other cases, given that we know there must be 12 5 S gene clusters in a G_2 nucleus, we conclude that the spots must indeed represent single clusters, and that the chromatids must be widely separated at these positions. We did not find significant pairing of spots in the G_1 cells - thus we may discount homologous pairing of interphase chromosomes in these cells.

We have shown a clear association of one or more clusters of 5 S genes with the nucleolus. This association seems to be irrespective of cell type or whether the cell is in G_1 or G_2. There is little previous information on the location of the 5 S genes during interphase in any species apart from *Drosophila melanogaster*. Steffensen (1977) and later Ananiev et al. (1981) reported a specific association of the 5 S locus (56EF) with the nucleolus in *Drosophila* salivary gland polytene chromosomes. However these observations were made on squashed preparations and no measurements or statistics were given. In contrast, Hochstrasser and Sedat (1987) analyzed the chromosome disposition in *Drososphila* polytene chrosomosomes in intact salivary glands and other tissues using 3-D optical microscopy and, of particular relevance to this study, the chromosome association with the nucleolus in prothorassic cells. Although the NOR-carrying X chrosomosome was, as expected, associated with the nucleolus, they found no nucleolar association of the 5 S locus.

It is tempting to speculate that the nucleolar association of 5 S gene clusters has a functional significance - for example, that the nucleolar associated genes are most actively transcribed, producing transcripts at the nucleolus where they are needed. It may be significant that we have not been able to visualize any significant nucleoplasmic labelling with anti-sense 5 S probe except after heat denaturation, which would reveal both genes and transcripts for labelling, although the nucleolar labelling was strong and clear; we might expect to see an accumulation of transcripts on and around actively transcribing genes. In fact, several investigators (e.g. see Lawrence et al., 1989; Xing et al., 1993) have seen distinct tracks emanating from transcribing genes, suggesting specific directed export paths. Although we have seen 'tails' emanating from 5 S clusters on occasion, with anti-sense labelling, this has only been after heat denaturation, which makes it impossible to determine whether the tail is due to the 5 S gene or transcript at the nucleoplasmic site; it seems most likely to be labelling of the genes. However, speculation about functional explanations for the nucleolar association raises some questions: for example, why should only some clusters be associated with the nucleolus and not all? This might imply that only these clusters are transcriptionally active, but there is no previous experimental evidence on this point. Although it is possible that only some of the 5 S gene clusters are transcriptionally active, there may be other reasons for lack of transcript labelling around the nucleoplasmic 5 S sites. One possibility is that the transcripts may be bound to associated proteins in such a way as to make them inaccessible to probe (although pronase digestion failed to reveal them), or the number of transcripts at the site of transcription may simply be too low for our detection system. However, we consider the latter possibility unlikely; the genes at these sites are easily detectable and it seems unlikely that the number of transcripts at and around these sites is less than the number of genes, even if only a small proportion of the genes in the clusters are transcriptionally active - enough 5 S transcripts must be produced to balance the nucleolar transcription of the rDNA. Other 'non-functional' explanations of the nucleolar association should also be considered. For example, specific structural aspects of the chromosomal organization might be important; two of the pairs of 5 S clusters are close to the telomeres and may thus have a greater tendency to be near the nuclear envelope where most of the telomeres are located (Rawlins et al., 1991). However, this would presumably make location of these clusters at the nucleolar periphery less likely. Another possibility might be that, during nucleologenesis, the NOR region expands as the nucleolus becomes active, and may 'sweep out' a region of the nucleus, collecting chromosomal regions in its path at the nucleolar periphery. If this were the case we should expect a correlation of nucleolar size with the number of nucleolar associated 5 S clusters. We have tested this statistically with Spearman's rank correlation coefficient on our data, and have shown that there is no such correlation - small nucleoli are likely to have as many clusters as large ones.

With anti-sense 5 S label, under a variety of conditions (heat denaturation, undenatured, pronase digestion) we have shown a characteristic cage-like network within the nucleolus. Its clear, reproducible and often very symmetrical appearance suggest that this represents an underlying pattern of organisation within the nucleolus. The structure is very similar to the organization of a nucleolar matrix antigen described by Corben et al. (1989). The 5 S transcript is known to associate with the growing pre-ribosomal particle at a very early stage, possibly even before the end of transcription of the 45 S pre-rRNA (Hadjiolov, 1985), and the labelling we have shown therefore probably represents all stages of pre-ribosomal processing and maturation. This is conventionally thought to occur in the granular component of the nucleolus, but there has been little study of the localization within the nucleolus of pre-ribosome processing. The granular component is generally assumed to fill the regions of the nucleolus not occupied by the other ultrastructural components (fibrillar centres and dense fibrillar component etc.) rather than having a defined structure itself, but if the 5 S transcript labelling we have shown does correspond to granular component or a part of it, this implies a much higher degree of structural organization than has been previously demonstrated.

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