INTRODUCTION

During the last ten years numerous studies have demonstrated that the cell nucleus is not randomly organized. On the contrary, the idea has emerged that both the nuclear components and the functions are typically distributed three-dimensionally for each step of the cell cycle (Manuelidis and Chen, 1990; Haaf and Schmid, 1991; Van Driel et al., 1991; Raska and Dundr, 1993; Spector, 1993). This concept has benefitted greatly from the development of new specific probes and of new techniques for their visualization, at both the ultrastructural and the optical level (Manuelidis and Borden, 1988; Puvion-Dutilleul et al., 1991; Thiry, 1993).

Until now, 3-D information has been obtained more easily and more frequently at the optical level by using wide-field microscopes or confocal microscopes equipped with software designed for de-blurring the images, for volumic reconstruction and for 3-D imaging (Carter et al., 1993; Hulspas and Bauman, 1992).

The usefulness of such methodologies is illustrated in the following examples. By using in situ hybridization (ISH) techniques for the location of various genes, a specific 3-D redistribution of the chromosomes may be identified during the different steps of the cell cycle with a typical profile for each subdomain (Vourc’h et al., 1993). Moreover, a speckled organization of sites of transcription was seen after immunolabeling of splicing factors, after ISH location of poly(A)^+ RNA (Carter et al., 1993) and after incorporation of Br UTP (Jackson et al., 1993).

SUMMARY

The relative three-dimensional co-location of RNA polymerase I (RPI) and DNA was studied using confocal laser scanning microscopy during interphase and all the steps of mitosis in human cancerous cells.

For each step of the cell cycle, immunolabeled RPI molecules and DNA specifically stained with chromomycin A3 were simultaneously imaged at high resolution through numerous optical sections. Then, all the data obtained were used to generate transverse sections, anaglyphs and volumic representations, which are all prerequisite approaches to a representative study of the three-dimensional organization of the nucleolus and the mitotic chromosomes. Our results indicated that in the interphase nuclei, in which DNA is organized as a regular 3-D network, RPI was present within numerous irregular spheres arranged as several twisted necklaces. During metaphase, RPI labeling was segregated into pairs of spheres and typical crescent-shaped structures; both were centrally located within the set of chromosomes. During anaphase and telophase, a typical central and symmetric arrangement of labeled structures was systematically seen among the decondensing chromosomes, arranged as a regular cylinder and as a hollow half-sphere, respectively. This typical 3-D organization of structures containing RPI relative to DNA is another strong example of the non-random organization of the genome during interphase and mitosis.

Key words: RNA polymerase I, DNA, confocal, 3-D reconstruction, interphase, mitosis

INTRODUCTION

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New probes and new modes of visualization have also added basic data to our knowledge of the most prominent nuclear organelle, i.e. the nucleolus. One nucleolus is a volume in which 200 to 500 rDNA genes are transcribed at a high rate for the synthesis of ribosomal particles (Hadjiolov, 1985; Warner, 1990). Numerous studies have been performed at the ultrastructural level to elucidate the precise location of rDNA genes, rRNA transcripts and proteins of the transcriptional machinery relative to the five nucleolar components, namely fibrillar centers, the dense fibrillar component, the granular component, the interstices and DNA (Goessens, 1984). Although a definite view of the molecular organization has not emerged from these studies (Derenzini et al., 1990; Scheer et al., 1993; Hernandez-Verdun, 1991; Jordan, 1991; Thiry et al., 1991; Schwarzacher and Wachtler, 1993), it is clear that the nucleolus is strictly organized relative to the steps of rRNA synthesis and processing (Puvion-Dutilleul et al., 1991; Hozak et al., 1994).

One example of this organization that is typical is shown by the immunolocation of an important nucleolar molecule: RNA polymerase I (or RPI). Immunolocation performed at the optical level reveals that RPI is restricted to many spheres
arranged as several necklace-like structures (Scheer and Rose, 1984; Haaf et al., 1991). At the ultrastructural level each sphere appears as a fibrillar center (Scheer and Rose, 1984).

A non-random organization of the nucleolar components during mitosis is also well documented. Therefore, rDNA genes are located in secondary constrictions, where they remain associated with several nucleolar proteins such as Ag-NOR proteins (Babu and Verma, 1985; Ploton et al., 1987b; Robert-Fortel et al., 1993), nucleolin (Gas et al., 1985), RPI (Scheer and Rose, 1984; Jimenez-Garcia et al., 1989; Haaf et al., 1991), and Upstream Binding Factor (Roussel et al., 1993).

Our goal was to investigate the 3-D organization of the nucleolus during interphase, and of its subcomponents during mitosis, relative to DNA using confocal microscopy. This study was performed within well-preserved cells that had been immunolabeled for RPI molecules and in which DNA was stained with chromomycin A3, a fluorochrome for DNA that needs no RNase pre-treatment for specific staining (Rigaut et al., 1991).

The 3-D co-location of the two labelings (RPI and DNA) was studied after taking numerous optical sections for each step of the cell cycle and then by obtaining both transverse sections and anaglyphs, and applying new software developed for the presentation of data in 3-D (Lucas et al., personal communication).

MATERIALS AND METHODS

Cell culture

KB cells derived from an epidermoid carcinoma were purchased from the American Type Culture Collection (Rockville, MD) and were grown at 37°C on coverslips 22 mm in diameter and placed in minimum essential medium (Sigma, St Louis, MO) containing 10% complement-depleted fetal serum, 2 mM L-glutamine, 100 i.u./ml penicillin and 100 mg/ml streptomycin.

Fixation and immunolabeling

Cells on coverslips were rinsed in phosphate buffered saline (PBS; 0.1 M, pH 7.2), then fixed in 3% paraformaldehyde in PBS containing 1% Triton X-100 for 4 minutes. Cells were placed in 10% normal goat serum (NGS, J. Boy, Reims) in PBS for 30 minutes, then incubated with a rabbit polyclonal antibody against the 42 and 24.6 kDa subunits of the RPI molecules (a gift from K. Rose) diluted 1:200 in PBS. This second antibody was revealed with streptavidin goat antibody (donkey anti-rabbit; Amersham) diluted 1:100 in PBS. After rinsing in PBS, the cells were incubated with a biotinylated goat anti-rabbit antibody (maximal excitation 445 nm; maximal emission 580 nm). The second laser was a 0.3 mW HeNe laser, producing one line at 543 nm to excite Texas Red specifically (maximal excitation 596 nm, maximal emission 610 nm). In order to obtain images that are perfectly registered we used a new filter-block that allows simultaneous observation of chromomycin A3 (DNA) and Texas Red (RPI) fluorescence. This filter-block is composed of a dicroic mirror at 560 nm and one long-pass filter emission at 570 nm. For each position of the z-stepping motor, one image of DNA and one of RPI were alternately or simultaneously obtained on one or two photomultipliers. Perfect registration was checked with multi-labeled spheres (Multispeck-Multispec-Trivial Fluorescence Microscopy Standards, Molecular Probes, Eugene, Oregon). For each nucleus (interphase) or cell (mitosis) the z-series (containing between 16 and 50 optical sections) of both labelings was obtained at a pitch of 0.3 µm. A minimum of five different cells were examined for each phase (interphase and phases of mitosis). In all cases fluorescence was obtained under the following conditions: (a) the pinhole in front of the detectors was minimally open (i.e. 0.6 mm); (b) an average of five images (Kalman filter) recorded at a speed of 2 seconds for 512×384 pixels was sufficient to obtain images with a good signal-to-noise ratio and to avoid bleaching; (c) the scanning zoom varied from ×4 to ×6. Under these conditions the pixel size varied from 0.065 to 0.044 µm, being near the Nyquist criterion (0.055 µm for a 1.4 NA and a wavelength of 550 nm).

Processing of the images

Images were stored on optical disks. Series of images were pre-processed with the Comos and Thruview software packages (Bio-Rad, UK) to modify contrast and to produce extended focus, transverse sections, rotation, stereo pairs, anaglyphs or a merging of the two labelings.

For 3-D reconstruction we used a software package developed in our group on a SUN 4 workstation (Sun Microsystems Inc., Mountain View, CA).

By the end of the treatment, the numerised volume appeared as a parallelepiped with three visible faces. On the upper one is presented the projection of all the optical sections for both labelings as numerised by the confocal microscope. On the two other faces are shown two orthogonal projections of the transverse sections. Conceived as a manner of visualization, this module is based on a first ray-tracing algorithm, where only the hidden sides are treated. Its aim is to map the projection derived from the three cardinal axes onto the visible sides of the voxel cube. Capable of modeling the physical properties of a light source and matter, as well as their interaction, ray-tracing is a technique that attaches itself to the inverse path of a light ray. For this, a screen, in the form of a table of a number of pixel per lines by a number of lines, as well as the associated geometry (position of the eye, view point reference, tilt axis, focal length and projection mode), is defined. Following this, a ray from the eye was passed through the center of the pixel in order to sample the scene. At the mapping module level, the scene is made up of an object and a punctual light source. Thus this 3-D representation, which allows one to see three angles of view of the total projections of the two labelings, gives an impressive image, at a glance, of the 3-D organization of DNA and RPI labelings.

Finally, images were printed on a video-printer (UP-5000, Sony-Japan).

RESULTS

Interphase

One optical section with DNA (blue) and RPI (red) labelings is shown in Fig. 1A. DNA appeared as clusters with a cord-like structure in some parts of the nucleus. Numerous areas without DNA appeared as holes whose size and shape were...
irregular (size varied from 0.5 to 2 µm for the smallest ones). The largest one corresponded to one nucleolus where both perinucleolar and intranucleolar clumps of DNA were identified. The RPI labeling appeared as numerous dots with diameters varying between 0.2 and 0.5 µm.

The extended focus of both labelings is shown at a higher magnification in Fig. 1B. This view demonstrated that DNA was organized as a regular cord of 0.4 µm in diameter, arranged throughout the whole volume of the nucleus as a complex 3-D network, the meshes of which corresponded to the ‘holes’ without DNA, as observed in single optical sections.

The extended focus also allowed us to characterize the RPI labeling present in the nucleolus in terms of real size, shape and total number of positive structures.

Thus RPI labeling appeared as numerous spheres with diameters varying from 0.2 to 0.6 µm. These spheres, of which there are around 50 for the largest nucleolus, frequently constitute necklace-like structures. The three yellow arrows in Fig. 1B indicate the three transverse sections shown in Fig. 1C, which are necessary to interpret the relative arrangement of DNA and RPI labelings within the nucleolus. Thus (in Fig. 1C) the nucleolus was always limited by a continuous sheet of DNA (perinucleolar DNA), from which several strands project within the nucleolus (intranucleolar DNA). Some of these strands touched some of the spheres of RPI; however, no sphere of DNA was detected overlapping RPI labeling (this fact can be verified by looking at this figure with the green glass of a pair of red and green spectacles, necessary for the visualization of anaglyphs). The spheres of RPI labeling were dispersed throughout all parts of the nucleolus.

Observation of the anaglyph (Fig. 1D) of the RPI labeling with red and green spectacles reveals that spheres of RPI belonged to at least five, more or less extended, necklace-like structures, frequently twisted and distributed throughout the whole thickness of the nucleolus. Finally, the observation of both labelings in the parallelipedic representation (see Fig. 4A) indicated that this nucleus is an ellipsoidal structure (around 15 µm in diameter and 4 µm thick) with RPI labeling being typically centred in the thickest part.

Prophase

The volumic representation of one cell during prophase (see Fig. 4B) showed that the number of RPI positive structures was greatly decreased (around 20 structures with a size varying from 0.3 to 0.7 µm). A preferential location of this labeling in the central part of the nucleus (relative to the thickness) was also identified. Two types of RPI structure were identified: the first ones were in close contact with condensing chromosomes, whereas the second were in the space between chromosomes.

During this step the nucleus was still clearly delimited. It was an ellipsoid around 12 µm in diameter and 3 µm thick. Condensing chromosomes, which are 0.8 µm in diameter frequently appeared as contorted structures joining the upper and the lower faces of the nucleus.

Metaphase

During this phase, the set of chromosomes appeared as an ellipsoid, which was positioned either transversely or in front view relative to the optical axis of the microscope. The latter is shown in Fig. 2.

One optical section is shown in Fig. 2A. Chromosomes, which were contained within a circle of 14 µm in diameter were optically cut either longitudinally or transversely. The former were more frequently located at the periphery of the set of chromosomes and were radially arranged, whereas the latter seemed to be more centrally located. Chromatids were around 0.7 µm in diameter and were frequently curved. The labeling of RPI was very limited and appeared as several granules 0.3 µm in diameter, located either in close contact with or inside chromosomes.

All the chromosomes appeared on the extended focus (Fig. 2B). The deepest ones (relative to the observer) were shown with a limited brightness, whereas the more proximal ones were the brightest.

Several points concerning the relative organization of the chromosomes appeared in this view. Firstly, all of the chromosomes seemed to be radially oriented relative to the set that is around 14 µm in diameter. Secondly, a great number of chromosomes were grouped in the more central part of the set and constituted a sphere with a diameter of around 8 µm.

Thirdly, the other chromosomes, less numerous, were present within a ring 3 µm wide, positioned at the outside of the former group. In this ring, individual chromatids, 3 to 4 µm long, were frequently observed.

Using this extended focus, RPI labeling appeared as two doublets of spheres (0.4 µm each) and two elongated structures (0.6 and 0.8 µm long). Typically, this labeling was confined to the more central part of the set of chromosomes i.e. within a circle of 3 to 4 µm in diameter.

The three yellow arrows in Fig. 2B indicate the level of the three transverse sections shown in Fig. 2C. On these transverse sections, the position of the chromatids relative to the set of chromosomes appeared clearly.

Taking the z-axis as a reference, one can deduce that chromatids were perpendicular, oblique or parallel to the z-axis. This confirmed that they were arranged radially within the set of chromosomes.

The co-location of DNA and of RPI labeling on the transverse sections indicated that RPI was integrated within the chromosomes. Moreover, the RPI structures present on the second and third sections now appeared as rounded and elongated masses, respectively. Comparison of this view with that of Fig. 2B indicated that these two structures were both crescent-shaped and orthogonally arranged relative to each other.

The 3-D shape and arrangement of the RPI labeling identified on the anaglyph are shown in Fig. 2-D. Positive structures appeared as four spheres and two crescent-shaped ones arranged in two orthogonal orientations.

Finally, the simultaneous 3-D representation of the two labelings (Fig. 4C) clearly summarizes all the data previously observed. The set of chromosomes was an ellipsoid in which chromosomes were arranged radially. The central part of this ellipsoid contained the majority of chromosomes, whereas the outer ring contained less chromosomes, but with larger arms. These were preferentially arranged in the plane of the larger axis of the ellipsoid. Finally, RPI structures appeared as ovoid and crescent-shaped masses, typically located within a central ellipsoid.
Fig. 1. Co-location of DNA (blue) and RPI (red) (A, B, C) and 3-D location of RPI (C) during interphase: (A) an optical section showing areas of DNA. Some cord-like structures appear (arrow); areas without DNA are also shown (+). Clumps of nucleolar DNA (arrowheads) are seen at the periphery and within the nucleolus. RPI labeling is constituted of numerous spheres (open arrow). (B) Extended focus of 15 optical sections showing that DNA is organized as a cord 0.4 µm in diameter (arrows). RPI labeling appears as about 50 spheres (open arrow). Yellow arrows indicate the positions of the three transverse sections shown in C. (C) Transverse sections showing clearly the position of the RPI labeling relative to DNA within the whole thickness of the nucleolus. Some clumps of DNA touch RPI spheres (arrowheads). (D) Anaglyph showing the 3-D repartition of the RPI labeling only (to be observed with red and green spectacles). The spheres belong to five twisted and contorted necklaces. Bar, 1 µm.
Fig. 2. Co-location of DNA (blue) and RPI (red) (A, B, C) and 3-D location of RPI (C) during metaphase: (A) an optical section showing chromosomes in longitudinal (arrow) or transverse section (arrowhead). RPI labeling is located at the periphery (small open arrow) or inside the chromosomes (large open arrow). (B) Extended focus of 37 optical sections. Deepest chromosomes have a limited brightness. The majority of chromosomes are grouped in the more central part of the set, whereas some chromatids are clearly visible at the periphery of the set (arrow). RPI labeling appears as two doublets of spheres (small open arrows) and two elongated structures (large open arrows), both located in the central part of the chromosome set. The three yellow arrows position the transverse sections shown in C. (C) On transverse sections the set of chromosomes appears as an ellipsoid (large axis in the horizontal position) in which chromosomes are oriented horizontally, obliquely or vertically (arrows 1, 2, 3, respectively). RPI labeling appears as spheres (small open arrow) or as elongated structures (large open arrow). (D) Anaglyph of RPI structures showing their relative 3-D arrangement. Bar, 1 µm.
Late anaphase

Optical sections through this cell during anaphase were performed parallel to the longer axis of the mitotic spindle. Fig. 3A shows an optical section of both DNA and RPI performed in the medium plane of the two sets of chromosomes. Each set appeared as a chambered structure 6 µm in diameter with a typical concave shape, in which the concave chamber was turned towards the mitotic pole of the cell. The thickness of the wall, the diameter of the chamber and its depth were, respectively, around 1 µm, 4 µm and 2 µm. The RPI labeling appeared as regular spheres with various sizes and arranged at the border of the DNA.

On the image of the extended focus (Fig. 3B) the typical chambered structure of the two sets of chromosomes was clearly seen. In each set, the RPI labeling appeared typically as two large spheres (0.5 µm in diameter) and 3 or 4 smaller ones (0.3 µm in diameter). These spheres were preferentially located near or within the closed part of the chambered sets of chromosomes.

Transverse sections (Fig. 3C), performed at the level of the yellow arrows in Fig. 3B, confirmed that each chromosome set was a hollow structure. From these transverse sections, and from Fig. 3A,B it can be deduced that each set of chromosomes resembled a hollow half-sphere with its open side turned towards the cell pole. Its wall, which was composed of more or less fused chromosomes, was smoother on the interior side. Opposite, near the exterior side, chromosomes that were arranged as two or three rings were still evident. The length of the arm was frequently around 3 µm with a diameter of 0.8 µm.

The structures containing the RPI labeling were typically located on the closed side of the hollow half-sphere and more frequently on the internal border of the DNA. The observation of the anaglyph (Fig. 3D) showed a striking similarity in the size and 3-D arrangement of the RPI structures in the two sets of chromosomes. For each set, the two largest spheres were located at some distance from each other, whereas the 3 or 4 smallest ones were located in a more central position.

The observation of the simultaneous 3-D representation of DNA and RPI labelings (Fig. 4D) confirmed the hollow half-sphere shape of the two sets of chromosomes and the non-random position of RPI positive structures. It also appeared that the telomeres were not fused at that stage. This view also confirmed the symmetric arrangement of the RPI positive structures in the two sets of chromosomes, as well as their preferential central location.

Late interphase

During this phase (Fig. 4E) each set of chromosomes still appeared as a chambered structure of around 8 µm at its largest diameter. Its wall was thicker than at anaphase (around 2 µm) and the depth and diameter of the concave chamber were around 1 µm and 3 µm, respectively. The width of the chromosomes is 0.8 µm. For RPI, 6 or 7 positive structures with sizes ranging from 0.3 µm to 0.5 µm were still visible.

As seen on transverse sections oriented at 90°, the RPI labeling was still centrally located within the chromosome set and preferentially at the border of the chamber. Finally, as for late anaphase, the symmetric arrangement of these structures is striking.

Early interphase

During this phase, the progeny nuclei were typically spherical, as observed in Fig. 4F, with a diameter around 10 µm. The number of RPI-positive structures was greatly increased (around 15 per nucleus). Moreover, some of these structures were arranged as necklaces, which were preferentially located within the more central region of the nucleus.

DISCUSSION

Recent investigations of the three-dimensional organization of the nucleus have frequently been performed using a confocal laser scanning microscope (CLSM) for many reasons (Hulspas and Bauman, 1992): firstly, CLSM is a non-invasive technique that respects the three-dimensional integrity of the cell; secondly, CLSM allows optical sections to be obtained with a much higher resolution and contrast than that with conventional microscopy; and thirdly, CLSM allows the simultaneous visualization of several markers and consequently it facilitates their three-dimensional co-location.

All these features are of primary importance for the location of nuclear proteins relative to DNA within three-dimensionally, well preserved cells, as in the present study.

DNA location

We chose chromomycin A3 (CA3), because its specific staining of DNA does not need a deleterious RNase digestion, contrary to propidium iodide, for example. Moreover, CA3 is easily excited with the 457 nm line of an argon laser (Rigaut et al., 1991) and gave images with higher contrast than with mithramycin, even after cytochemical labeling (Ploton et al., 1994). Finally, bleaching was extremely slight and this is of major importance for the achievement of numerous optical sections, having both a high signal to noise ratio, as well as a high resolution.

Immunostaining

The simultaneous use of a short fixation and a permeabilization procedure allowed the preservation of both the antigenicity and the 3-dimensional organization of the nucleus. This was assessed in our study by the positive labeling of nucleolar components such as fibrillar centers, by the preservation of the fine 3-D organization of the DNA network and by a normal nuclear ultrastructure.

Potential limitations of CLSM imaging

It is necessary to interpret the images very carefully when the three-dimensional co-location of several labelings is concerned in CLSM (Brelje et al., 1993). It is well known that there may be several reasons for the aberrations or misrepresentation of the images, both in the x-y plane and along the z-axis. These causes may be: (1) the change of filter sets and/or the use of two lasers, which are both required for the imaging of several labelings; (2) spherical or chromatic aberrations; (3) the depth of the object investigated relative to the coverslip.

In our case, it was unnecessary to change the filter set, because we used a filter specially built for the simultaneous imaging of CA3 and Texas Red. Moreover, the alignment of the two lasers, as well as the registration of the images, were
checked by using fluorescent beads loaded with several fluo-
rochromes. On the other hand, we maximally limited chromatic
and spherical aberrations by using a planapochromat objective.
Finally, we measured the total thickness of the cells investi-
gated to ensure that it never exceeded 30 µm, i.e. the thickness
at which aberrations are limited (Majlof and Forsgren, 1993).

In summary, we can consider that misrepresentation of the
images and aberrations were maximally limited and that the
demonstration of proximity (or absence of proximity) of the
two labelings in all directions of the volume is effective.

**Interphase nucleus**

**DNA**

We demonstrated that DNA is organized as a regular 3-D
network constituting a thread 0.4 µm in diameter. This network
is similar to the one described by Manuelidis and Chen (1990)
after propidium iodide staining of neurons. This network is
more easily visible within cells with a high transcription
activity and is considered as one of the highest levels of comp-
tection within interphase nucleus (Manuelidis and Chen,
1990). Its systematic observation may be due to the high tran-
scriptional activity and the short doubling time (around 24
hours) of the cancerous cells we investigated. The meshes of
the DNA network constitute a contorted volume in which RNA
transcription, processing and exportation could probably take
place (Spector, 1993).

The images of nucleolar DNA (peri- and intra-nucleolar
DNA coming in close contact with RPI spheres) closely match
the previous ultrastructural specific location of DNA with osmium ammine complex (Derenzini et al., 1984). This technique also allowed several authors to identify spheres of decondensed DNA located at the level of fibrillar centers (FC) and dense fibrillar components (Derenzini et al., 1990). Sur-
prisingly, we never identified spheres of DNA which would
have been superimposed on spheres of RPI labeling. Our
findings indicate either that there is no DNA within the FC or
that the observation of decondensed DNA is impossible in our
conditions. This more probable hypothesis could be explained
either by a level of fluorescence under the limit of detection of
our microscope (Pawley, 1990) or by the quenching of flu-
orescence due to the numerous proteins associated with rDNA
(Rost, 1992).

**RPI**

Several important features of the RPI labeling were assessed.
Firstly, the labeling is segregated as spheres, which are, very
probably, fibrillar centers (FC) as earlier identified at the
electron microscopy level (Scheer and Rose, 1984; Thiry et al.,
1991). Secondly, our study showed that the spheres present
various diameters (from 0.2 to 0.6 µm) within the same
nucleolus. From ultrastructural studies it has been known for
a long time that nucleoli may contain one very large FC
(around one or two µm in diameter) and several smaller ones
(0.3 µm in diameter) for defined cell types (Ploton et al.,
1987b). However, very frequently, the size of the FC is smaller
(in the range of 0.2 to 0.5 µm) and it is quite impossible to
measure it precisely on ultrathin sections (Keiding and
Andersen, 1992). Due to its high resolution in the x-y plane,
CLSM appears to be a very efficient tool to identify such small
differences which might correspond to different molecular
compositions or to various numbers of transcriptional units
(see below). Thirdly, we definitely demonstrated that RPI
positive spheres are systematically arranged as large twisted
necklaces. However, contrary to previous studies describing
these extended structures after RPI labeling, it was necessary
neither to artificially modify the 3-D organization of nucleolar
structures by using nucleoside analogues such as DRB (Scheer
and Benavente, 1990; Haaf et al., 1991), nor to smear the cells
(Haaf et al., 1988) to illustrate them. This points to an
important possibility for CLSM, which is to investigate large
volumes and to demonstrate the three-dimensional continuity
of structures such as FC in the necklaces. The examination of
transverse sections and of anaglyphs clearly indicates that
necklaces are contorted extended structures within the whole
volume of the nucleolus. More or less naturally extended
necklaces were previously identified within the nucleoli of
various cell types after the immunolocation of RPI (Haaf et al.,
1991) and UBF (Roussel et al., 1993), after location of rRNA
transcribing sites (Jackson et al., 1993) or after Ag-NOR
staining (Ploton et al., 1994). From these studies and from
other results we obtained previously (Ploton et al., 1987a), we
can hypothesize that the transcriptionally active part of one
nucleolus is organized as necklaces of subunits (or beads), each
constituted by one central sphere (one FC), surrounded by
several connected loops of dense fibrillar component (DFC).
Each bead of the necklace could correspond to one functional
unit as suggested by Derenzini et al. (1990). At the present
time, several hypotheses exist concerning the number of rDNA
genes per bead. Each FC could contain either one rDNA gene
(Scheer and Benavente, 1990; Haaf et al., 1991, Schwarzacher
and Wachtler, 1993) or at least 5 active rDNA genes (Hozak
et al., 1994). On a working model, Hozak et al. (1994)
proposed that 6 transcription units are gliding at the surface of
one FC and that RPI molecules are only located within the FC
and absent from the DFC, which is in agreement with
immunolocation (Scheer and Rose, 1984 and the present
work). According to Haaf et al. (1991), each necklace is a
decondensed nucleolar organizer region (or NOR), initially
segregated as a cluster of rDNA genes at the level of one
secondary constriction during metaphase. This means that in
KB cells, 3 to 5 NORs are maximally active at any one time
during interphase. Finally, the continuity between several
beads of one necklace could be due to some parts of the DFC
as observed at the electron microscopy and confocal levels
(Ploton et al., 1987a, 1994; Robert-Fortel et al., 1993) or to a
nucleolar skeleton (Hubert and Bourgeois, 1986; Hozak et al.,
1994).

**Mitotic cells**

Our study allowed us to obtain a complete timing of the 3-D
organization: firstly, of the mitotic chromosome set; and
secondly, of the structures containing RPI molecules and their
relative 3-D location. More particularly, bearing in mind that
during mitosis RPI molecules remain associated with rDNA
genes (Matsui and Sandberg, 1985), at the level of nucleolar
organizer regions (Scheer et al., 1984; Haaf et al., 1988, 1991),
which are only located on the secondary constriction of the
human acrocentric chromosomes (Babu and Verma, 1985), the
labeling of RPI molecules may also serve to localize: the rDNA
genes; the NORs; and, approximately, the centromeric regions
of the acrocentric NOR-bearing chromosomes.
Fig. 3. Co-location of DNA (blue) and RPI (red) (A, B, C) and 3-D location of RPI (C) during late anaphase: (A) an optical section showing that each set of chromosomes is a concave-chambered structure turned towards the mitotic pole. RPI labeling appears as spheres (open arrow) located at the border of DNA. (B) Extended focus of 30 optical sections. In each set, the RPI labeling is constituted of two large spheres (large open arrows) and 3 or 4 small spheres (small open arrow), which are both located near the closed part of the chambered set of chromosomes. The three yellow arrows indicate the positions of the transverse sections shown in C. (C) Transverse sections demonstrating the chambered shape of the two sets of chromosomes. The interior edge of the DNA is smooth (arrows) whereas, on the exterior side, chromatids are still visible (arrowheads). RPI spheres are typically located on the more internal side of the chromosomes sets (large open arrows). (D) Anaglyph of the RPI-labeled structures showing their identical 3-D repartition in the two sets. Bar, 1 μm.
Fig. 4. Volumic representation of DNA and RPI labelings during each phase of the cell cycle. For each phase, 3-D data are presented as a parallelepiped with three visible faces showing each the extended focus of DNA and RPI labelings simultaneously. DNA and RPI labelings are shown in grey and in white, respectively. (A) Interphase: necklaces of RPI labeling are clearly identified and are mainly located in a central position within the nucleus. (B) Prophase: the number of RPI spheres decreases. (C) Metaphase: RPI labeling is distributed near the center of the chromosome set. Note the typical radial arrangement of the chromosomes. (D) Late anaphase: the symmetric 3-D organization of the RPI spheres is very apparent. Telomeres of chromosomes are not fused. (E) Late telophase: the central position of the RPI spheres is clear within the two re-forming nuclei. (F) Early interphase: the number of RPI spheres increases within the two spherical progeny nuclei.
Mitotic chromosomes

During metaphase, the chromosome set clearly appears as an ellipsoid, in which all the chromosomes are radially aligned. The chromosomes are arranged as several concentric rings and their telomeres are located at various distances from the center of the metaphase plate. From the RPI labeling, it can be deduced that the position of the acrocentric chromosomes is not random. It is undoubtful that the NORs (and the centromeres) are grouped in a limited volume in the proximity of the center of the chromosome set. All these results corroborate the results of Mosgöller et al. (1991) obtained after 3-D reconstruction of metaphase plates observed on serial ultrathin sections.

Concerning the complex shape of the two chromosome sets during the phases of mitosis our results corroborate to a large extent those of Welter et al. (1985), which were performed with scanning electron microscopy (or SEM) on isolated chromosome sets for various phases of mitosis. Our results give highly complementary data to that study although both approaches are different. Thus, if the resolution was much higher in the study of Welter et al. (1985), the investigation by SEM necessitated the isolation of the chromosome sets which could have modified their 3-D organization. In our case, the chromosomes conserve their original position and shape. Moreover, the use of fluorescent labeling opens up the possibility of obtaining data from all parts of the chromosome. This is not the case with SEM. Our study confirmed that the complex 3-D shapes of chromosome sets after metaphase are as follows: a cylinder, a hollow sphere, a flattened disc and a sphere, during anaphase, early and late telophase and early interphase respectively (Welter et al., 1985).

Our data indicate that during anaphase the centromeric regions of the NOR-bearing chromosomes are located in the closed part of the cylinder where chromatids are fused. This, again, is indicative of a high 3-D organization of the chromosome set. Moreover, from transverse sections, it can be ascertained that most of the telomeres are in the leading position during late anaphase and that chromosomes are fused only in the vicinity of their centromeric region. Finally, the fusion of chromosomes will proceed through the telomeres during the late phases of mitosis (Welter et al., 1985).

NORs

During prophase, the disaggregation of the nucleolus leads to a decrease in the number of positive spheres and to the absence of any diffuse labeling between chromosomes, contrary to what is observed for other nucleolar proteins (Gautier et al., 1992). Two hypotheses can be proposed for the decrease in the number of stained structures: firstly, a great part of the RPI molecules could have been solubilized during prophase and were possibly undetectable (Haa et al., 1991); or secondly, the interphase necklaces could have been compacted to the mitotic NORs, but without a loss of RPI molecules. This last hypothesis is ascertained by the work of Conconi et al. (1989) demonstrating that the extended structure of previously active rDNA genes (with their associated RPI molecules) remains unchanged during mitosis. However, due to the fact that the fluorescence level cannot be directly related to the quantity of RPI molecules (Rost, 1992), neither of these two hypotheses can be confirmed or excluded from our observations.

The high resolution of the images indicated two different types of metaphasic NORs: spherical and crescent-shaped. These results corroborate those obtained after Ag-NOR staining observed on ultrathin (Ploton et al., 1987b) or thick sections (Ploton et al., 1987a) or by confocal microscopy (Robert-Fortel et al., 1992) and are another indication of the heterogeneity in the size of metaphasic NORs (Babu and Verma, 1985).

During anaphase, transverse sections indicate a typical location for NORs along the border of DNA, as was earlier found for argyrophilic components in other cells (Ploton et al., 1987b).

As previously shown (Jimenez-Garcia et al., 1989) the labeling of RPI in the two sets of chromosomes during anaphase and telophase are almost identical. We were also able to demonstrate that the NORs are of various sizes (within each set of chromosomes) and that their position is not at random in the x, y and z directions. This typical arrangement is also indicative of a high 3-D organization of the NOR-bearing chromosomes during these phases.

Moreover, contrary to what we found after Ag-NOR staining, numerous and small RPI-positive structures were never found during these phases (Ploton et al., 1987b). This is another indication of the existence of two pools of nucleolar structures present during telophase (Jimenez-Garcia et al., 1989): the first are NORs and contain rDNA genes, RPI, nucleolin and Ag-NOR proteins, while the second are numerous, small (0.2 µm) aggregates of nucleolar proteins (without RPI), which fuse with NORs to constitute prenucleolar bodies when functional RPI molecules are present (Scheer et al., 1993). Finally, the decondensation of the clusters of rDNA genes may explain the rapid recovery of several necklaces typically distributed within the central part of the progeny nuclei.

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