

# Nuclear Spreads: I. Visualization of Bipartite Ribosomal RNA Domains

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**Abstract.** Visualization of nuclear architecture is key to the understanding of the association between RNA synthesis and processing. This architecture is obscured by the high density of components in most nuclei. We have developed a method of spreading nuclei and nucleoli that reduces overlap of weakly associated components. Strong interactions among nuclear components are not disrupted by this method. Spread nucleoli remained structurally distinct and functionally competent in ribosomal RNA synthesis. Nascent

ribosomal RNA colocalized with RNA polymerase I and fibrillarin, a protein required for processing of ribosomal RNA. Colocalization of nascent transcripts and fibrillarin was seen in nucleoli spread over several microns, suggesting a strong interaction. These data suggest that nucleoli are superassemblies of bipartite domains, each composed of a ribosomal RNA synthesis center tightly associated with areas likely to be involved in ribosomal RNA processing.

THE interphase nucleus carries out most of the important reactions in the metabolism of RNAs: RNA synthesis (Lewin, 1980), processing (Moore et al., 1993; Sollner-Webb and Mougey, 1991), and transport to the nuclear pore complex (NPC)<sup>1</sup> for exit to the cytoplasm (Mehlin et al., 1992). Elements of nuclear architecture mediate a tight temporal and topological orchestration of these processes. mRNAs are processed cotranscriptionally. (Osheim et al., 1985; Beyer and Osheim, 1988; LeMaire and Thummel, 1990; Greenleaf, 1993; Bauren and Wieslander, 1994). The identification of the sites of synthesis and processing, however, has not been achieved (Fakan et al., 1971; Fu and Maniatis, 1990; Carter et al., 1991, 1993; Huang and Spector, 1991; Zamore and Green, 1991; Spector et al., 1991; Zhang et al., 1992; Xing et al., 1993; Jackson et al., 1993; Wansink et al., 1993; reviewed in Spector, 1993a,b). The prevailing view is that nucleoplasmic architecture physically compartmentalizes the sites of mRNA synthesis and processing (Smetana et al., 1963; Fakan et al., 1976; Sass and Pederson, 1984; Fakan et al., 1986; Spector et al., 1991; Huang and Spector, 1991; Carter et al., 1993; Xing et al., 1993; Jackson et al., 1993; Wansink et al., 1993). This compartmentalization has been used to explain why mRNAs artificially driven by RNA polymerase III (pol III) cannot be properly spliced (Sisodia et al., 1987). A physical link be-

tween synthesis and processing is consistent with the observed recruitment of splicing factors to sites of new transcription in cells transfected with plasmids containing parts of the rat tropomyosin gene or cells infected with adenovirus 2 (Jimenez-Garcia and Spector, 1993). Moreover, treatment of cells with agents that inhibit pol II transcription disrupts the structural organization of splicing factors (Spector et al., 1983; Carmo-Fonseca et al., 1992; reviewed in Spector, 1993a). It is clear that domains housing both mRNA synthetic and processing factors are likely to exist; however, to date, these domains have not been identified clearly.

The topological relation between synthesis and processing has also been investigated for RNA pol I transcripts in nucleoli (Gerbi et al., 1990). Nucleoli are subnuclear compartments containing the ribosomal RNA (rRNA) genes (Heitz, 1931; McClintock, 1934; Rittosa and Spiegelman, 1965; Sollner-Webb and Mougey, 1991), the rRNA synthetic and processing machinery (Scheer and Rose, 1984; Rendon et al., 1992; Kass et al., 1990), and the ribosome assembly machinery (Busch and Smetana, 1970). Nucleoli have been shown by electron microscopy to have discernible regions or components: the fibrillar centers, the dense fibrillar components, and the granular regions (reviewed in Stahl, 1982, and in Spector, 1993a). Fibrillar centers appear as circular structures in thin sections and they are usually completely surrounded by and immediately adjacent to dense fibrillar components. The granular regions are commonly found surrounding the dense fibrillar components. The biochemical processes occurring within each of these regions have not been established convincingly. Some investigators using autoradiography and electron microscopy have concluded that rRNA is synthesized in the periphery of fibrillar centers in areas that usually abut on dense fibrillar components (Thiry

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1. *Abbreviations used in this paper:* BrU, 5-bromoUTP; NPC, nuclear pore complex; pol, polymerase; PBS++, PBS supplemented with 0.4% (wt/vol) Tween 20 and 5 mg/ml ovalbumin; rRNA, ribosomal RNA; RT, room temperature.

and Goessens, 1991; Puvion-Dutilleul et al., 1991; Thiry, 1992; reviewed in Spector, 1993a). Nevertheless, others using similar techniques point to the dense fibrillar components as the sites of synthesis (Granboulan and Granboulan, 1965; Schöfer et al., 1993). Recently, Hozák et al. (1994) have used 5-bromoUTP (BrU) incorporation and immunoelectron microscopy, and concluded that synthesis occurred in the dense fibrillar components. Based on immunofluorescence staining of processing factors, rRNA processing is likely to take place in fibrillar centers and/or dense fibrillar components (Ochs et al., 1985). Recently, detection of incorporation of bromoUTP into rRNA by immunofluorescence revealed that synthesis occurs within distinct nucleolar foci, however, it is not possible to discern whether these are fibrillar centers or dense fibrillar components (Jackson et al., 1993; Wansink et al., 1993).

Conditions required to observe nuclear structures are usually incompatible with RNA synthesis and processing, and extracts capable of recapitulating these nuclear functions have no apparent architecture (Dignam et al., 1983; Manley et al., 1980). The density of the cell nucleus presents at least two other problems for visualization of functionally relevant interactions: stacking of structures obscures view of distal objects and complicates the interpretation of proximal objects. Moreover, in this crowded organelle, colocalization of two abundant components can be attributed to chance rather than to a functional association. To overcome these difficulties in the study of the architecture of transcription and processing, we developed a method to spread nuclei and thus decrease the density of the material available for observation. The spread nuclei were shown to have many components of nuclear architecture such as U snRNP speckles and nucleoli. Moreover, the spread nuclei were capable of carrying out rRNA synthesis, which colocalized with pol I in spherical bodies. Each spherical body was tightly associated with a region containing fibrillar, which can be taken as a marker of rRNA processing. This association predicts the existence of rRNA synthesis and processing domains, and we posit that the nucleolus is the superassembly of many of these domains.

## Materials and Methods

### Antisera and Antibodies

A mouse anti-bromoU monoclonal antibody was purchased from Boehringer Mannheim Corp. (Indianapolis, IN). A mouse anti-fibrillar monoclonal antibody (72B9) was described in Reimer et al. (1987). Human antisera with specificity against fibrillar were obtained from Dr. S. Baserga (Yale University, New Haven, CT) and have been described (Ochs et al., 1985). Rabbit antiserum to B lamins was a gift from Dr. R. Goldman (Northwestern University, Evanston, IL). Human antisera with specificity against RNA pol I were obtained from Dr. J. Craft (Yale University). Human antiserum (AW) with specificity against Sm determinants (U snRNP-associated proteins) was obtained from Dr. J. Keene (Duke University); this Sm-type of serum has been described (Lerner et al., 1981). Fluorescein- and rhodamine-conjugated secondary antibodies with specificity for human or mouse IgGs were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) divided into aliquots for single use, and stored at  $-80^{\circ}\text{C}$ .

### Cells and Tissue Culture

CV-1 (African green monkey kidney) cells were grown in MEM supplemented with 10% fetal calf serum, 20 mM Hepes, glutamine, and peni-

cillin/streptomycin, without pH indicator dye. For most experiments, cells were passed on to flamed No. 1 coverslips ( $2.2 \times 2.2$  cm) at very low density ( $\sim 100$  cells/coverslip). Cells were permitted to attach for  $\geq 24$  h after passage before experiments were conducted.

### Cell Lysis and Nuclear Spreads

Coverslips with CV-1 cells attached were assembled into chambers over glass slides. Briefly, two parallel lines of silicone stopcock grease were formed  $\sim 1.5$  cm apart on a glass slide ( $2.5 \times 7.5$  cm) and fragments of No. 0 coverslip were used as spacers. Coverslips with cells were inverted and pressed onto the grease and spacers to form a chamber with open ends. The volume of the chambers was  $\sim 20$   $\mu\text{l}$ . The cells were washed with 200  $\mu\text{l}$  chilled PBS supplemented with 15  $\mu\text{g/ml}$  PMSF in 1% EtOH and 10  $\mu\text{g/ml}$  chymostatin, leupeptin, antipain, pepstatin in 1% (vol/vol) DMSO. Cells were lysed with 50  $\mu\text{l}$  of PBS supplemented 1% (wt/vol) Triton X-100 and protein inhibitors as described above. Incubation with lysis buffer was at room temperature for 30 s. Isolated nuclei were fixed at this stage as described below. Nuclei were swollen with 200  $\mu\text{l}$  of 1 M Tris- $\text{PO}_4$  (pH 9.1) or 1 M Tris-HCl (pH 8.8) at room temperature (RT) for 30 s to 2 min. Swollen nuclei were spread with 100  $\mu\text{l}$  of PBS supplemented with protease inhibitors. Nuclear spreads or isolated nuclei were fixed at RT for 5 min with 50  $\mu\text{l}$  of 2% (wt/vol) paraformaldehyde in PBS, which was made from a fresh stock of 20% paraformaldehyde buffered with NaOH. The paraformaldehyde was quenched with 50  $\mu\text{l}$  of 1 mg/ml Na borohydride incubated at RT for 5–10 min. Fixed spreads were washed with 100  $\mu\text{l}$  of chilled PBS with RNasin (1  $\mu\text{l}$  in 100  $\mu\text{l}$  of PBS) and with 400  $\mu\text{l}$  of chilled PBS supplemented with 0.4% (wt/vol) Tween 20 and 5 mg/ml ovalbumin (PBS++).

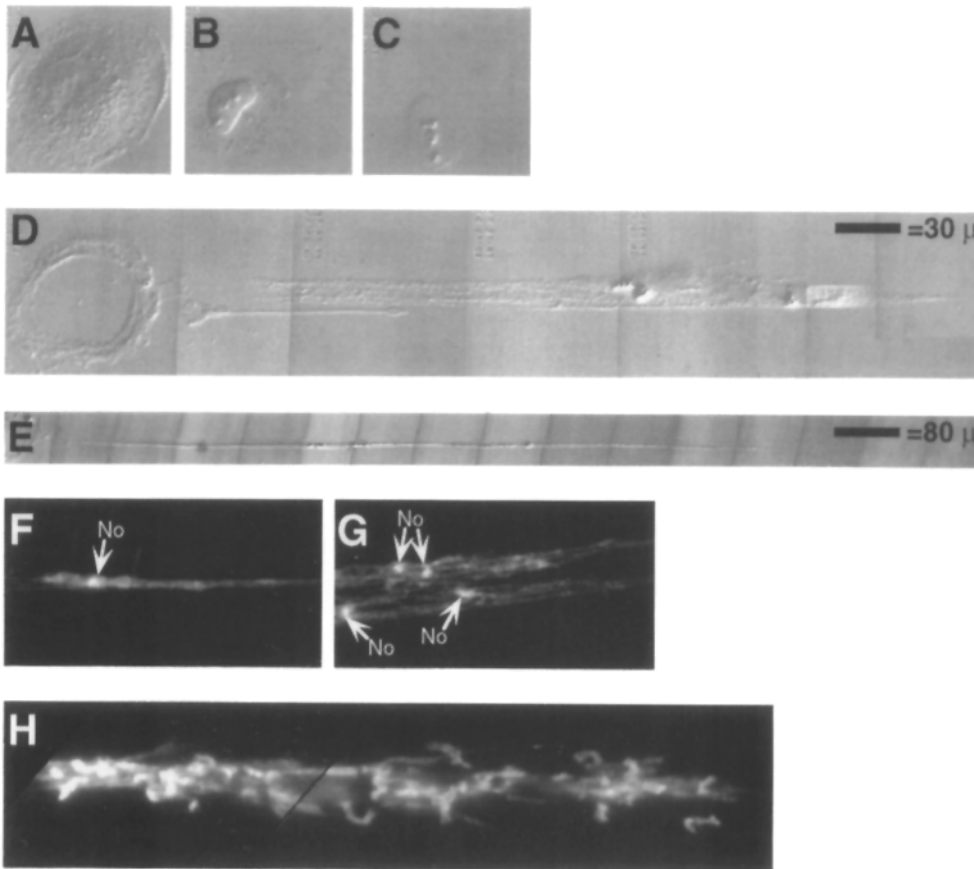
### In Situ Transcription Reactions

Nuclear spreads or isolated nuclei were washed with 100  $\mu\text{l}$  of Dignam buffer D (20 mM Hepes [pH 7.9], 0.2 mM EDTA, 20% (vol/vol) glycerol, 0.5 mM DTT supplemented with protease inhibitors, and 1  $\mu\text{l}$  of RNasin (Promega Biotech, Madison, WI [per 500  $\mu\text{l}$ ]) (Dignam et al., 1983). Spread nuclei were incubated with 50  $\mu\text{l}$  of transcription mix at  $37^{\circ}\text{C}$  for 10 s to 60 min. The transcription mix contained 600  $\mu\text{M}$  ATP, GTP, and GTP, 1 mM 5-bromoUTP (Sigma Immunochemicals, St. Louis, MO) 0.74 $\times$  buffer D (or 0.37 $\times$  buffer D when HeLa nuclear extract was included), 6.6 mM  $\text{MgCl}_2$ , 75 mM KCl, 10 mM creatine phosphate, and if indicated, HeLa nuclear extract to a final concentration of 37% (vol/vol). The transcription mix was supplemented with protease inhibitors and RNasin as described above. To quench transcription, the spread nuclei were washed with 100  $\mu\text{l}$  chilled PBS supplemented with RNasin and quickly fixed and washed as described above.

### Fluorescent Microscopy

The specific dilutions used for 1 $^{\circ}$  and 2 $^{\circ}$  antibodies and antisera are indicated in the figure legends. The anti-BrU antibody was always used at a dilution of 1:20 in PBS++. The monoclonal antibody to fibrillar was used at a 1:500 dilution in PBS++. Human antisera were used at dilutions of 1:1,000 in PBS++ and all secondary antibodies were used at 1:2,000 in PBS++. 1 $^{\circ}$  and 2 $^{\circ}$  antibody/antiserum incubations were at RT for 2 h in a dark humidified chamber. Antibody and antiserum incubations were terminated by washing with 400  $\mu\text{l}$  of PBS++. Before microscopic inspection, the chambers were washed with PBS supplemented with 50 mM DTT and sealed. Chambers were inspected immediately or stored at  $4^{\circ}\text{C}$  overnight in the dark.

Images were photographed using either an Axioskop (Carl Zeiss, Inc., Thornwood, NY) or a Photomicroscope (Nikon Inc., Melville, NY) and Tmax 3200 black and white film or 400 Ektachrome color film (Eastman Kodak Co., Rochester, NY) (Figs. 2, 4, and 8). Images were also collected with a cooled CCD camera (Star Camera; Photometrics Ltd., Tucson, AZ) and stored using the IPlab program. Background images were collected from microscopic fields adjacent to experimental images. The background was subtracted from images to correct for instrument variation in the fields. Whereas experimental values reached 4,000, background pixel values rarely exceeded 50. Negative pixel values were converted to 0, and the images were rescaled from 0 to 255. Images were assembled and printed using Canvas and Adobe Photoshop (Figs. 1, 3, 5–7, and 9). All computer manipulations were done with a Macintosh Quadra. Images were printed photographically or directly from the computer on a printer (Phaser II SDX; Tektronix, Inc., Beaverton, OR).



**Figure 1.** Spreading of CV-1 cell nuclei. (A) CV-1 cell visualized by video enhanced DIC microscopy. (B-E) Video-enhanced DIC micrographs. (B) Nucleus (isolated nucleus) of CV-1 cell that was lysed with PBS supplemented with 1% (wt/vol) Triton X-100 and protease inhibitors. Three nucleoli are visualized within the nucleus. Cytoplasmic material can be seen around the nucleus. (C) Swollen nucleus after treatment with 1 M Tris-Po<sub>4</sub> (pH 9.1). (D) Spread nucleus is seen spanning most of the panel, the ring-like structure to the left of the spread nucleus is the cell ghost. Three nucleoli can be seen within the spread nucleus. The scale in A-D is indicated by the bar in D. (E) A spread nucleus of ~1,000 μm in length. (F) Spread nucleus stained with Hoechst, this elongated fiber-like spreading was the most commonly observed. A nucleolus (No) is indicated. (G) Spread nucleus stained with Hoechst shown here as an example of a second form typically observed with

this method. The fibers in this nucleus have unraveled into more and finer component strands. Nucleoli (No) are indicated. (H) Spread mitotic nucleus showing that mitotic chromosomes were not elongated and sister chromatids remained associated. This image was reconstructed from three partially overlapping fields.

### Western Assays

SDS-PAGE and Western transfer procedures were performed under standard conditions. Proteins were transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) and detected using alkaline phosphatase-conjugated secondary antibodies (Promega Biotech) as described by the manufacturers.

### Results

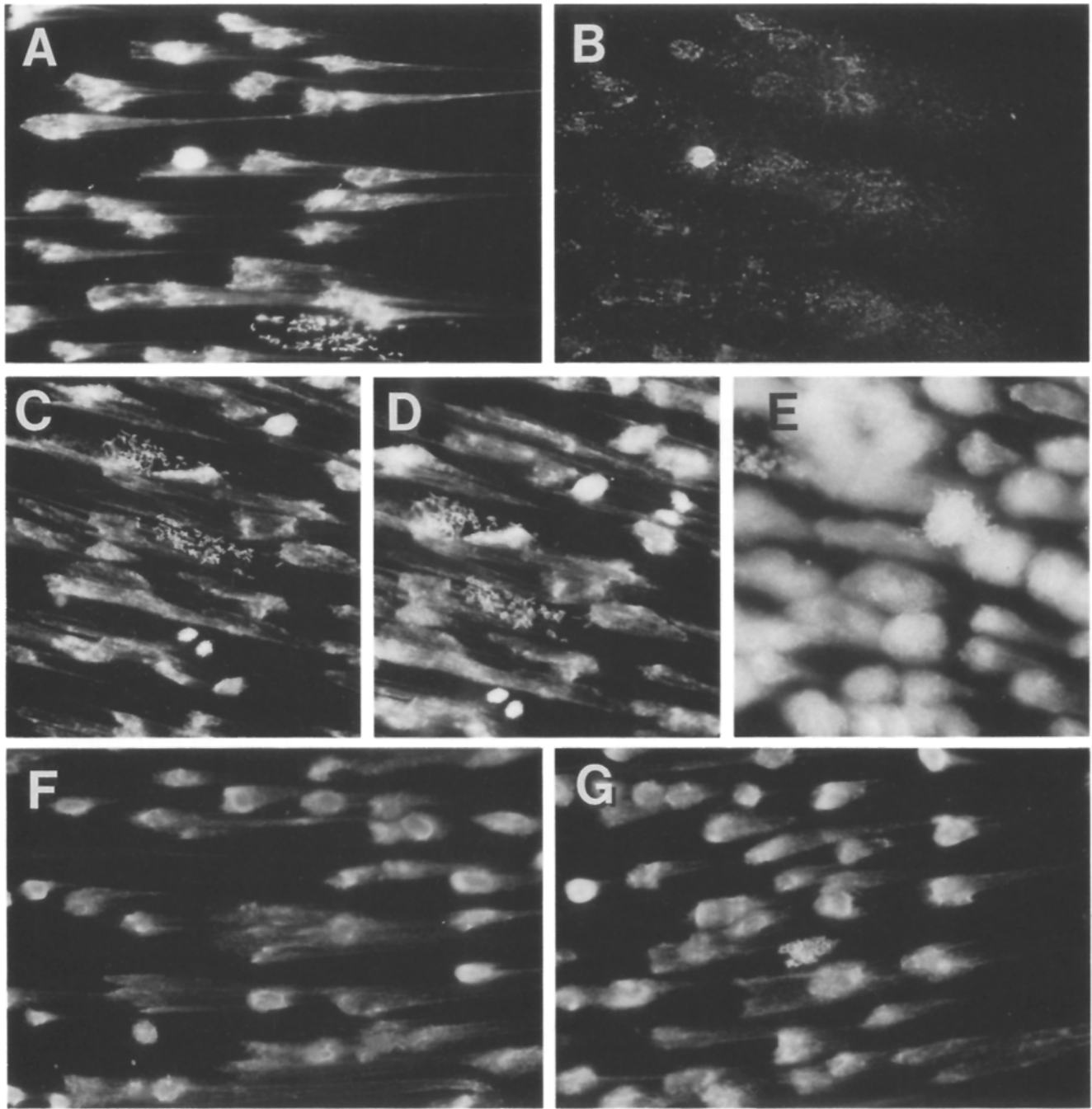
#### CV-1 Nuclei Can Be Extensively Spread

African green monkey kidney (CV-1) cells were grown to a sparse density on coverslips (Fig. 1 A). The coverslips were assembled on glass slides to form chambers where the solutions bathing the cells could be exchanged rapidly and efficiently. The cells were lysed in situ with PBS and 1% (wt/vol) Triton X-100 supplemented with protease inhibitors. The nuclei of CV-1 cells remained attached to the coverslips and were permeable to large molecules such as antibodies (Fig. 1 B, and data not shown). A 1-M Tris-Po<sub>4</sub> (pH 9.1) solution was used to swell the nuclei (Fig. 1 C). Swelling was documented by video-enhanced DIC microscopy and typical increases in all three linear dimensions were 1.3–2-fold. Both the contrast of the nucleus and the rigidity decreased dramatically. The swollen nuclei were spread by rapid flow of PBS, and the osmotic shift may contribute to the disintegration of the Triton-extracted nuclear envelope (Fig. 1 D).

Fluid shear forces resulted in spreading of nuclei into strands that were visualized by video-enhanced DIC microscopy and had a contrast slightly greater than microtubules in the same system. The spread nuclei were sometimes as long as 1,000 μm, thus ~50 times the length of the unspread nuclear diameter (Fig. 1 E). The length of time in the 1-M Tris-Po<sub>4</sub> buffer was critical; times <1 min gave very poor spreading and times >3 min resulted in disruption of all nuclear structures including nucleoli (see below). The spread nuclei were either incubated with components needed for transcription or directly fixed in 2% paraformaldehyde to determine the distribution of structural components. Fixation did not affect the structures observed in spread nuclei (data not shown).

The spread nuclei stained with Hoechst dye (Fig. 1 F). Spreading sometimes resulted in one predominant tail emerging from the nucleus (Fig. 1 F) or in a spectacular array of fibers of varying diameters (Fig. 1 G). Some of the structures that stained more intensely with Hoechst were visualized within spread nuclei (Fig. 1, F and G), were later morphologically and immunologically identified as nucleoli (see below). Mitotic chromosomes did not spread with this method and remained attached to one another by thin fibers that stained weakly with Hoechst (Fig. 1 H).

Spread nuclei were fragmented by incubating with 1 mg/ml DNase I for 10 s at room temperature (Fig. 2, A and B). Recoil of the fibers was often seen with DNase I treat-

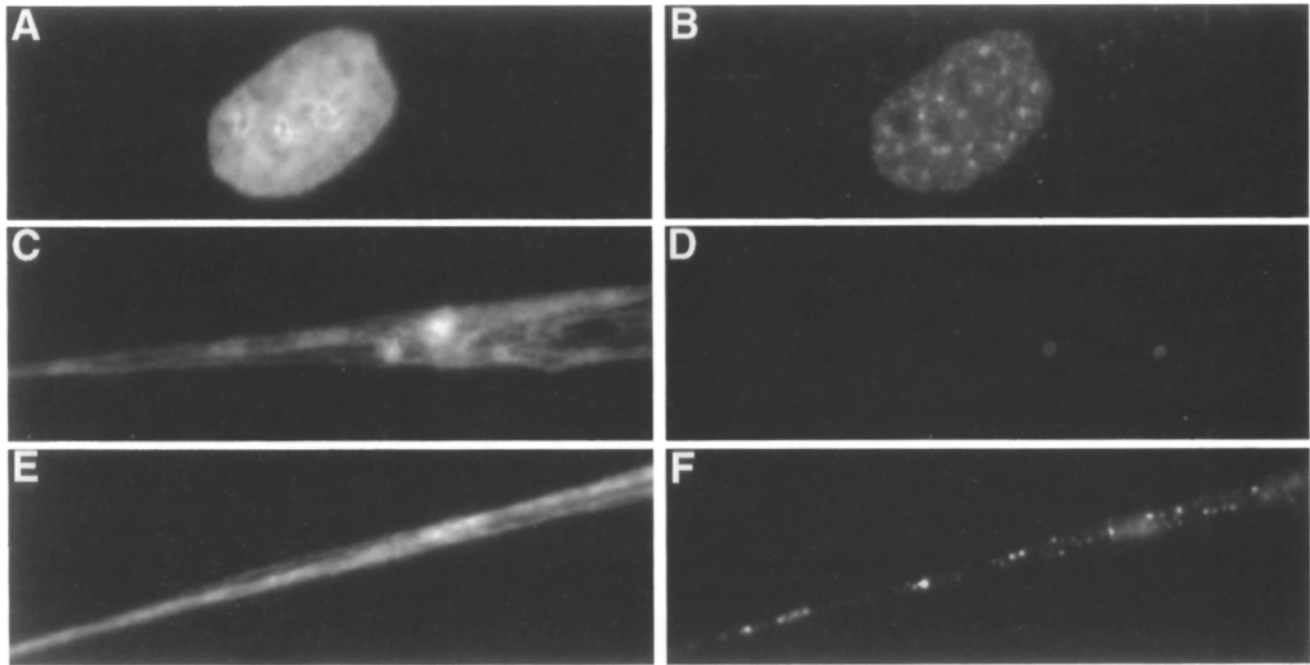


**Figure 2.** Spread nuclei are sensitive to DNase I and chymotrypsin, but not to RNase A. (A) Spread nuclei stained with Hoechst showing heterogeneity of spreading that was usually observed in dense cell populations. (B) The same spread nuclei treated with 1 mg/ml DNase I for 10 s at room temperature. This field mostly overlapped the field shown in A. (C) Spread nuclei stained with Hoechst. (D) The same spread nuclei as in C treated with 1 mg/ml chymotrypsin for 10 s at room temperature. (E) Same as D, now treated with 1 mg/ml chymotrypsin for 5 min at room temperature. (F) Spread nuclei stained with Hoechst. (G) The same spread nuclei as in C treated with 1 mg/ml RNase A for 5 min at room temperature.

ment, indicating that the fibers were stretched elastically. Although the spread nuclei were also sensitive to chymotrypsin treatment, the results was an expansion of the strands and general decondensation of the structure (Fig. 2, C-E). Treatment with 1.0 mg/ml of RNase A did not alter the structure of the strands (Fig. 2, F and G). These studies revealed that spread nuclear strands required both protein and DNA for their structural integrity.

#### *Elements of Nuclear Architecture in Spread Nuclei*

An anti-Sm serum, which reacts with common U snRNP proteins, stained speckles in isolated nuclei (Fig. 3, A and B). A diffuse nucleoplasmic staining was also visible and was coincident with Hoechst staining, except over nucleoli. Nonimmune sera did not stain isolated nuclei (data not shown) or spread nuclei (Fig. 3, C and D). Spread nuclei

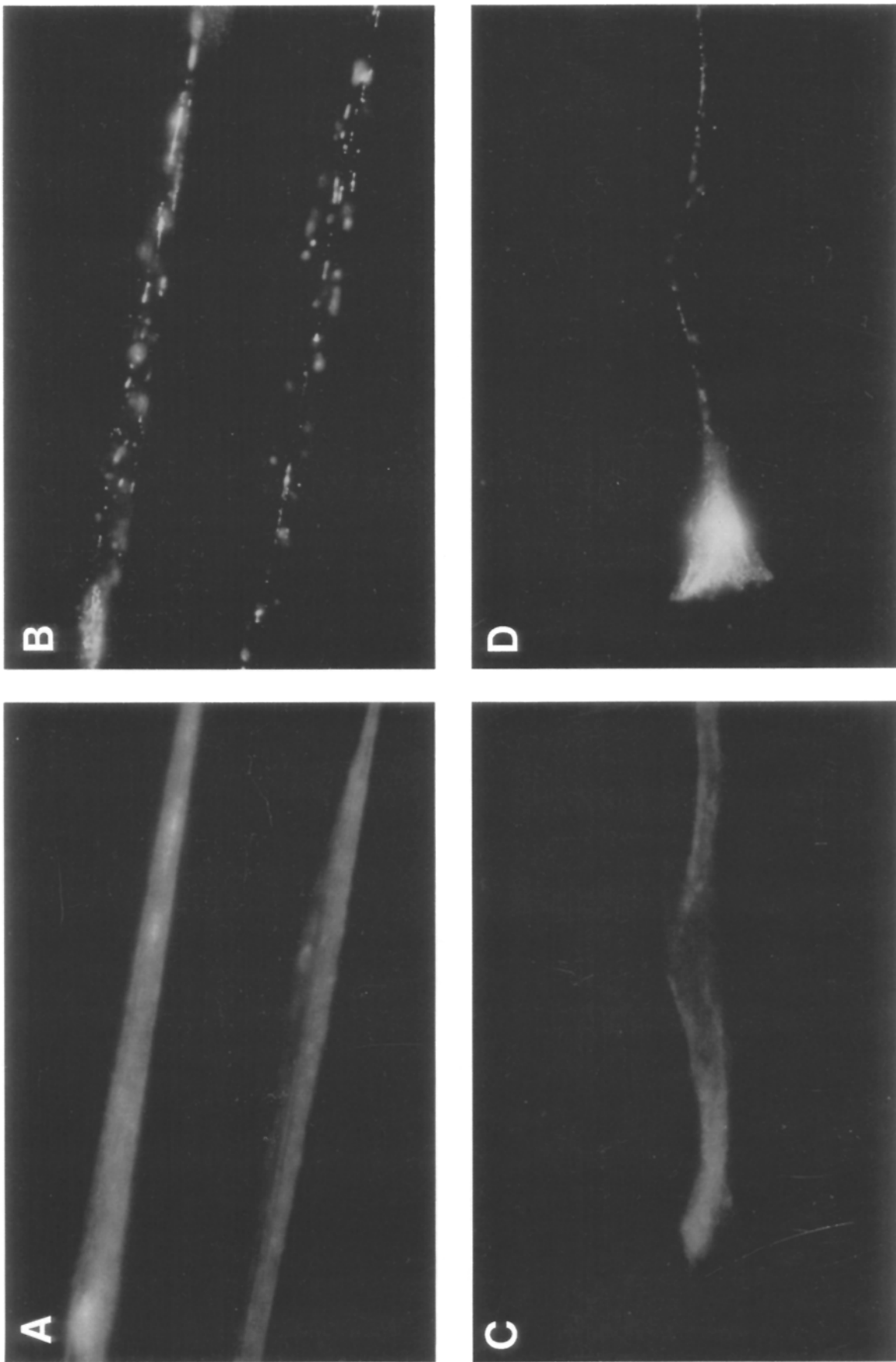


**Figure 3.** Nuclear U snRNPs in spread nuclei. CV-1 cells were lysed as described in Fig. 1, an isolated nucleus is shown in *A* and *B*. Nuclei were swollen and spread as described in Fig. 1; spread nuclei are shown in *C–F*. Isolated nuclei and spread nuclei were fixed with 2% paraformaldehyde and stained with antisera or antibodies and with Hoechst dye. (*A*) Isolated nucleus stained with Hoechst. (*b*) Same nucleus stained with anti-U snRNP (*Sm*) serum and a secondary anti-human IgG antibody coupled to rhodamine. The “speckled pattern” and a diffuse nucleoplasmic stain can be observed. Nucleoli are not stained by the *Sm* serum. It is possible that some of the speckles in the isolated nuclei are nuclear or coiled bodies (Brasch and Ochs 1992; Carmo-Fonseca et al., 1992; Lamond and Carmo-Fonseca, 1993). (*C*) Spread nucleus stained with Hoechst. (*D*) Same spread nucleus stained with nonimmune (normal) serum detected as in *B*. Low levels of background stain are observed. (*E*) Spread nucleus stained with Hoechst. (*F*) Same spread nucleus stained with anti-U snRNP (*Sm*) serum detected as in *B*. A spread “speckled pattern” was observed. (*G*) Proteins extracted from: HeLa nuclear extract (lane 1), CV-1 cells (lane 2), CV-1 isolated nuclei (lane 3), and CV-1 spread nuclei (lane 4) were subjected to SDS-PAGE and transferred to Immobilon membranes. *Sm* antigens were detected with the anti-U snRNP (*Sm*) serum used in *B* and *F* and a secondary anti-human IgG coupled to alkaline phosphatase. The closed arrowhead probably represents snRNP proteins B and B'. The open arrowhead identifies the 70-kD protein associated with the U1 snRNP. Molecular mass markers are the pre-stained markers from Amersham Corp. (Arlington Heights, IL).

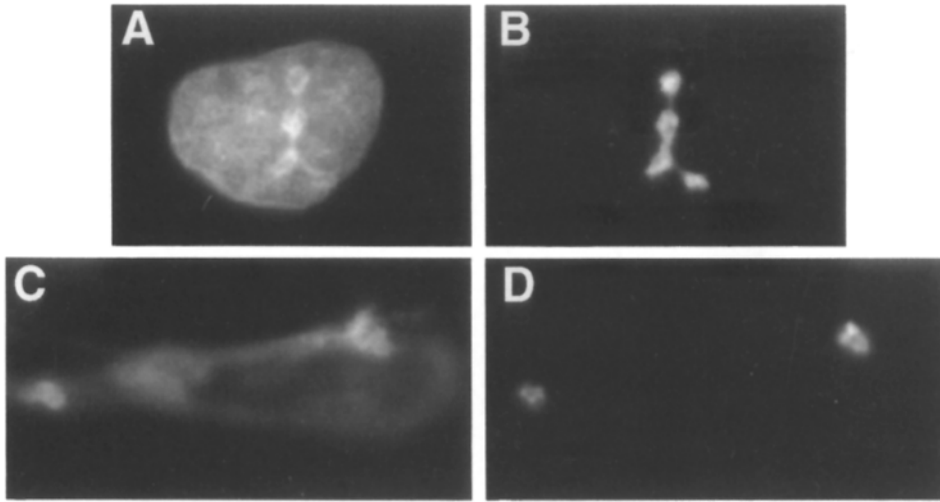
stained with this serum revealed speckles and diffuse staining within the regions stained with Hoechst (Fig. 3, *E* and *F*). The number of speckles remaining in spread nuclei varied, and in some cases, only very few (0–3) were detectable. These losses were also observed when CV-1 cells in suspension were treated with the lysis and spreading solutions. Analysis of proteins that remain associated with nuclei revealed that more than half of the *Sm*-reactive U snRNP-associated proteins were extracted by 1M Tris- $\text{PO}_4$  (Fig. 3 *G*).

When the spreads were stained with anti-lamin B antibod-

ies, a series of spots or short linear elements was observed along the Hoechst-staining strands (Fig. 4, *A* and *B*). This anti-lamin antibody stained primarily the nuclear envelope in isolated nuclei, and in partially spread nuclei, it showed a series of disconnected lines and spots that represented a fragmented nuclear lamina (data not shown). In highly spread nuclei, the lamin staining was often spaced as much as 10  $\mu\text{m}$  along the DNA-containing strands. In contrast, antikeratin staining was distinct from the Hoechst staining, although there was occasional overlap (Fig. 4, *C* and *D*).



**Figure 4.** Lamin-containing structures in spread nuclei. (A) Spread nuclei stained with Hoechst. (B) Same spread nuclei stained with anti-lamin antibody and a secondary anti-mouse IgG coupled to rhodamine. Lamin colocalized with Hoechst stain. (C) Spread nucleus stained with Hoechst. (D) Same spread nucleus stained with anti-keratin antibody detected as in B. The keratin was observed colocalizing with the cell ghost and bordering the spread nuclei, but not within it.



**Figure 5.** Nucleoli in spread nuclei. CV-1 cells were lysed as described in Fig. 1, an isolated nuclei is seen in *A* and *B*. Nuclei were swollen and spread as described in Fig. 1, a spread nucleus is shown in *C* and *D*. Isolated nuclei and spread nuclei were fixed with 2% paraformaldehyde and stained with antisera or antibodies and with Hoechst dye. (*A*) Isolated nucleus stained with Hoechst. (*B*) Same nucleus stained with anti-fibrillarlin serum and a secondary anti-human IgG antibody coupled to rhodamine. Four nucleoli are observed to be connected by fibrillarlin-containing structures.

Four or five spots in the nucleoplasm stained for fibrillarlin and are probably nuclear or coiled bodies (Brasch and Ochs, 1992; Carmo-Fonseca et al., 1992; Lamond and Carmo-Fonseca, 1993). (*C*) Spread nucleus stained with Hoechst. (*D*) Same spread nucleus stained with anti-fibrillarlin serum detected as in *B*.

### Spread Nucleoli

Fibrillarlin is thought to be involved in the processing of rRNA and can be used as a marker for the early rRNA processing machinery of the nucleolus (Tollervey et al., 1993; Baserga and Steitz, 1993). To specifically identify nucleoli, spread nuclei were stained with a monoclonal antibody and an antiserum that specifically recognize fibrillarlin. In isolated nuclei, these two reagents gave an almost identical staining pattern. The pattern of Hoechst stain was in many cases sufficient to localize nucleoli (Fig. 5 *A*), which could be definitively identified by the anti-fibrillarlin serum (Fig. 5 *B*). In this case, nucleoli appeared connected by fibers that stained with the anti-fibrillarlin serum; however, these connections were not always observed. In moderately spread nuclei, the nucleoli could be identified using Hoechst and stained specifically with anti-fibrillarlin antiserum (Fig. 5, *C* and *D*, respectively). Spread nucleoli appeared larger and less dense than nucleoli in isolated nuclei, which may have been caused by the loss of components.

An antiserum against pol I immunostained nucleoli in isolated nuclei (Fig. 6 *C*) and in spread nuclei (Fig. 6 *G*). In isolated nuclei, this serum and several other human anti-pol I sera also stained the nucleoplasm weakly (Fig. 6, *A* vs *C*). The isolated nuclei and the spread nuclei were also stained for fibrillarlin using a mouse monoclonal antibody (Fig. 6, *B* and *F*, respectively). The colocalization of fibrillarlin and pol I observed over most of the area of nucleoli in both isolated and spread nuclei argues that the microarchitecture of the nucleoli was retained after spreading (Fig. 6, *D* and *H*, respectively). It is clear, however, that the spread nucleoli were partially unraveled and subnucleolar components could be resolved in great detail.

### rRNA Transcription in Spread Nuclei

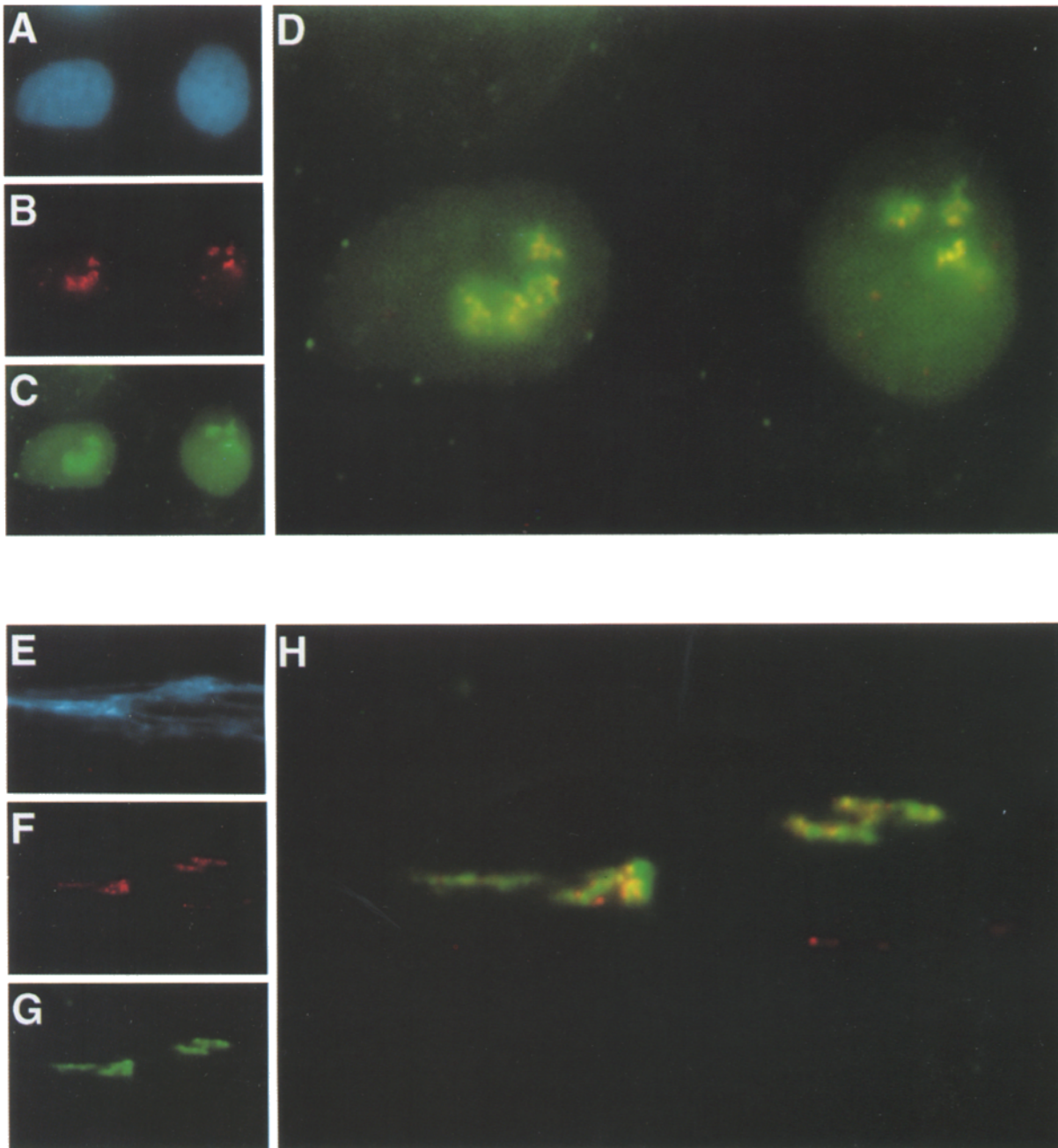
Isolated and spread nuclei were washed and incubated under conditions known to support *in vitro* transcription in nuclear extracts (Marciniak et al., 1990). The incorporation of BrU into RNA was detected *in situ* by indirect immunofluores-

cence with a mouse monoclonal antibody (anti-BrU) that recognizes brominated pyrimidines (Jackson et al., 1993; Wansink et al., 1993). In isolated nuclei (Fig. 7 *A*), the most dramatic incorporation of BrU into nascent RNAs was always found in nucleoli (Fig. 7 *C*). This is in contrast to Hožák et al., (1994), Jackson et al. (1993), and Wansink et al. (1993), who report weak nucleolar staining. Within nucleoli, nascent transcripts localized to distinct spherical bodies of  $\sim 0.5 \mu\text{m}$  in diameter (Fig. 7 *C*) (see also Jackson et al., 1993; Wansink et al., 1993). These spherical bodies appeared connected, giving the impression of "beads on a string" or necklaces reminiscent of patterns observed by others when staining for the transcription factor UBF (Zatsepin et al., 1993) or for argyrophilic proteins (Robert-Forstel et al., 1993) (see Discussion). Only a fraction of the area of the nucleoli was covered by these bodies. The spherical bodies staining with anti-BrU antibodies overlapped with much larger areas staining with anti-pol I (Fig. 7, *B-D*; see also Fig. 5). Pol I was detected throughout most of the nucleolus, although it appeared to be concentrated in the spherical bodies (Fig. 7 *B*).

Nucleoli in spread nuclei stained with anti-BrU antibody, indicating that these nucleoli were competent to synthesize rRNA (Fig. 6, *G*; see also Figs. 7 and 8). rRNA synthesis was detected in the same spherical bodies observed in isolated nuclei (Fig. 7 *F*). Pol I colocalized with nascent transcripts as was seen in isolated nuclei (Fig. 7, *F-H*).

Nucleolar BrU incorporation was temperature dependent, with much higher levels detectable after incubation at  $37^\circ\text{C}$  than at room temperature or  $4^\circ\text{C}$  (data not shown). Incorporation of BrU in spread nucleoli was enhanced by the inclusion of a HeLa cell nuclear extract in the transcription mix, but was not dependent on this (Fig. 8 and data not shown). The BrU incorporation was inhibited by the addition of  $10 \mu\text{g/ml}$  actinomycin D (Fig. 8, *F* vs *B* and *D*). Actinomycin D at concentrations as low as  $1 \mu\text{g/ml}$  inhibited nucleolar BrU incorporation, whereas  $1 \mu\text{g/ml}$  of amanitin had only a minimal effect (data not shown). RNase A treatment after transcription abolished the signal obtained with anti-BrU but





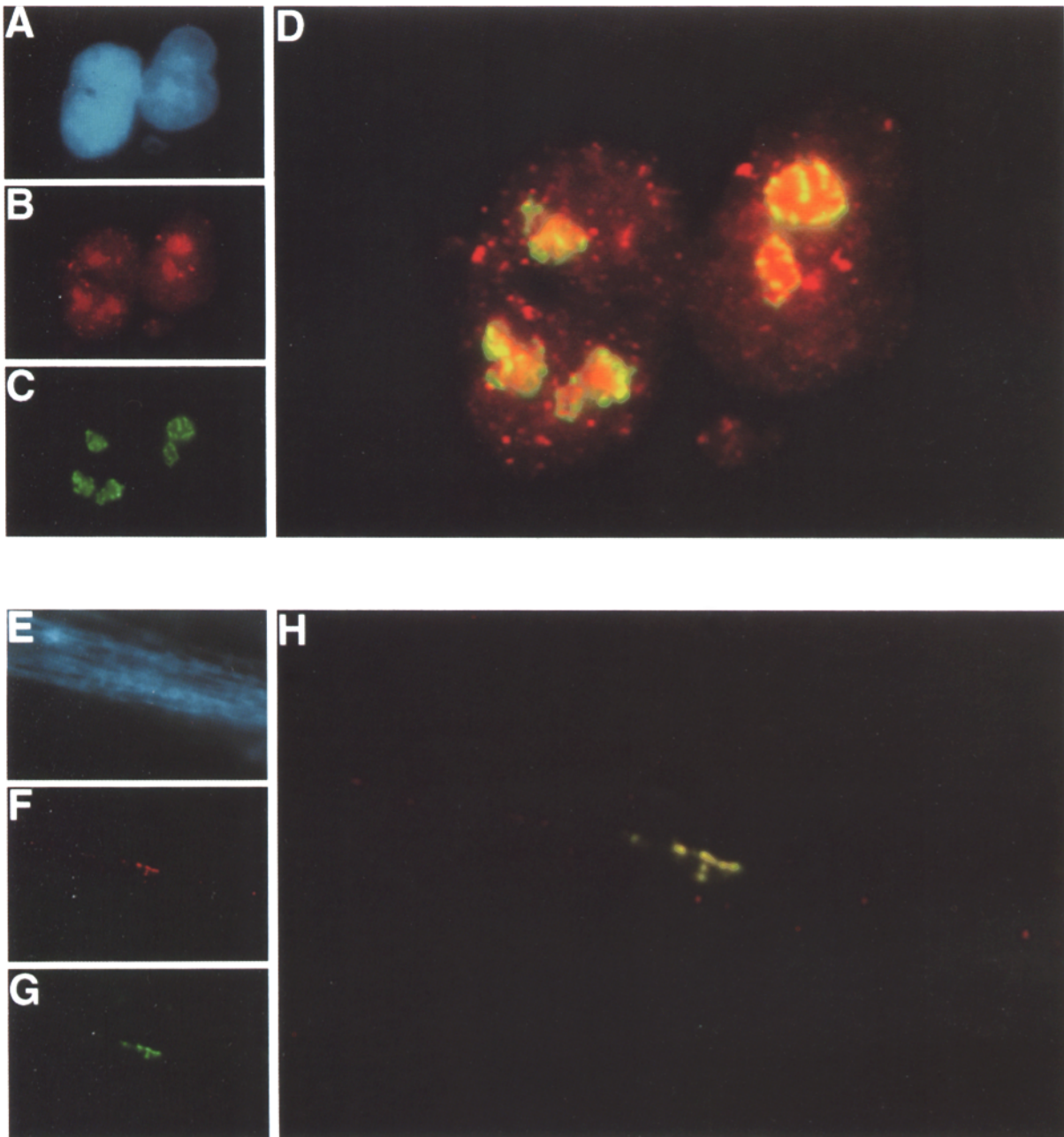
**Figure 6.** RNA polymerase I and fibrillarin colocalize in spread nucleoli. (A) Isolated nuclei stained with Hoechst. (B) Same nuclei stained with the 72B9 anti-fibrillarin monoclonal antibody and a secondary anti-murine IgG coupled to rhodamine. (C) Same nuclei stained with anti-RNA polymerase I (*pol I*) serum and a secondary anti-human IgG coupled to fluorescein. (D) Same nuclei showing overlap (yellow) of the fibrillarin and the pol I staining. (E) Spread nucleus stained with Hoechst. (F) Same spread nucleus stained with the 72B9 anti-fibrillarin monoclonal antibody and a secondary anti-murine IgG coupled to rhodamine. (G) Same spread nucleus stained with anti-pol I and a secondary anti-human IgG coupled to fluorescein. (H) Same spread nucleus showing overlap (yellow) of the fibrillarin and the pol I staining.

not with anti-fibrillarin antibodies (data not shown). These data indicated that nucleolar BrU incorporation in isolated and spread nuclei was equivalent to rRNA synthesis.

BrU incorporation was not detected in extranucleolar sites in either isolated nuclei or spread nuclei (Fig. 7). This was taken as an indication that pol II transcription was not taking place in these nuclei. An antibody against the largest subunit of RNA polymerase II failed to give any signal in spread

nuclei using immunofluorescence and Western blot analysis (data not shown). The same antibody gave a very weak signal in isolated nuclei. Spread nuclei incorporated BrU into many extranucleolar sites, however, provided that transcription reactions included HeLa nuclear extract. Extranucleolar staining was punctate, and the intensity of the staining was variable in different regions of a strand (Fig. 8, A-D). Extranucleolar RNA synthesis was inhibited by 10  $\mu\text{g/ml}$  ac-





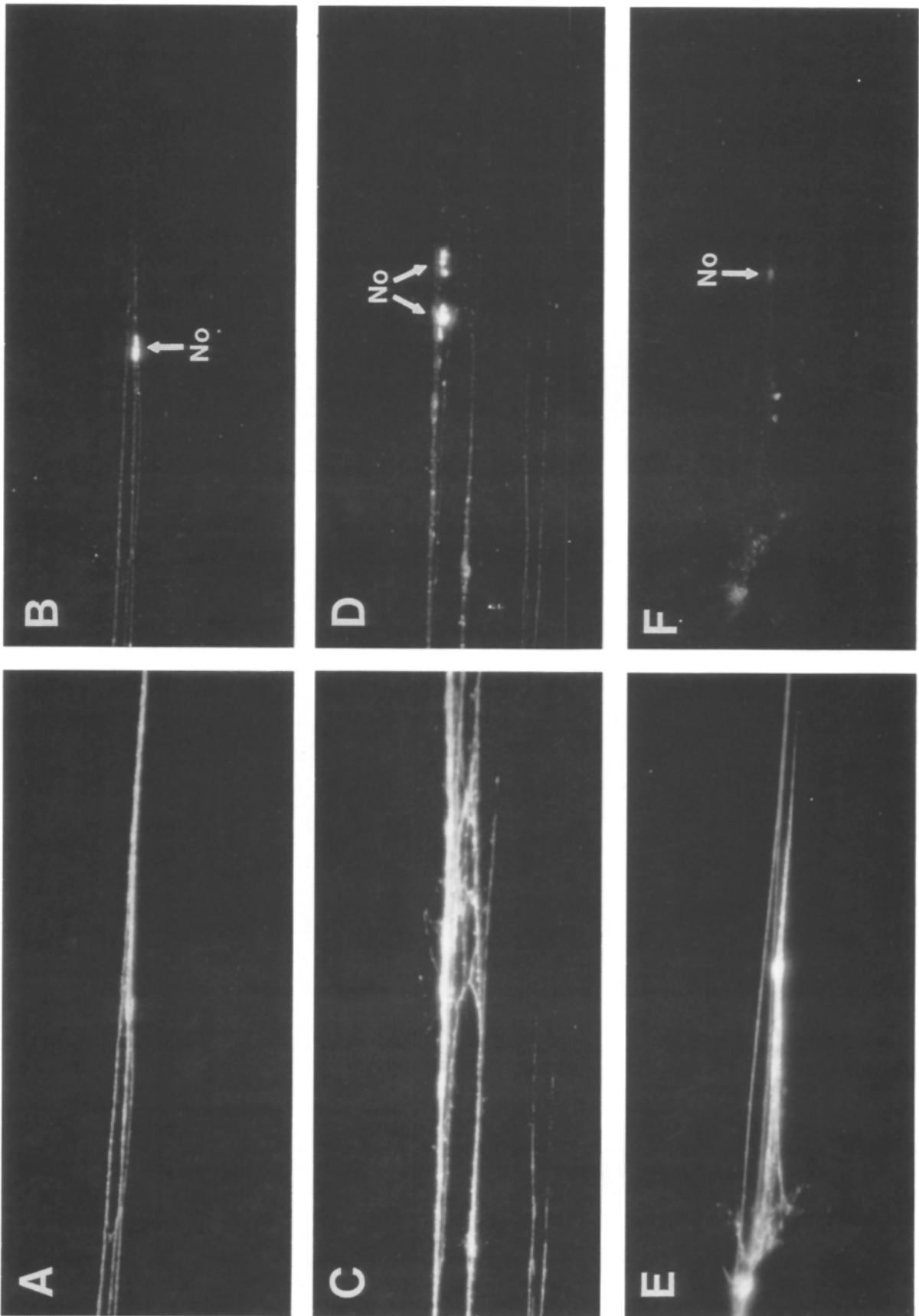
**Figure 7.** Transcription of rRNA in spread nucleoli. Isolated nuclei and spread nuclei prepared as described in Fig. 1 were washed in transcription buffer and incubated for 5 min at 37°C in this buffer supplemented with NTPs (UTP was replaced with BrU). Isolated nuclei and spread nuclei were washed and fixed with 2% paraformaldehyde and stained with antisera or antibodies and with Hoechst dye. (A) Isolated nuclei stained with Hoechst. (B) Same nuclei stained with anti-RNA pol I serum and a secondary anti-human IgG coupled to rhodamine. (C) Same nuclei stained with anti-BrU monoclonal antibody and a secondary anti-murine IgG coupled to fluorescein. (D) Same nuclei showing overlap (yellow) of BrU (rRNA synthesis) and pol I staining. (E) Spread nucleus stained with Hoechst. (F) Same spread nucleus stained with anti-RNA pol I serum and a secondary anti-human IgG coupled to rhodamine. (G) Same spread nucleus stained with anti-BrU monoclonal antibody and a secondary anti-murine IgG coupled to fluorescein. (H) Same spread nucleus showing overlap (yellow) of BrU (rRNA synthesis) and pol I staining.

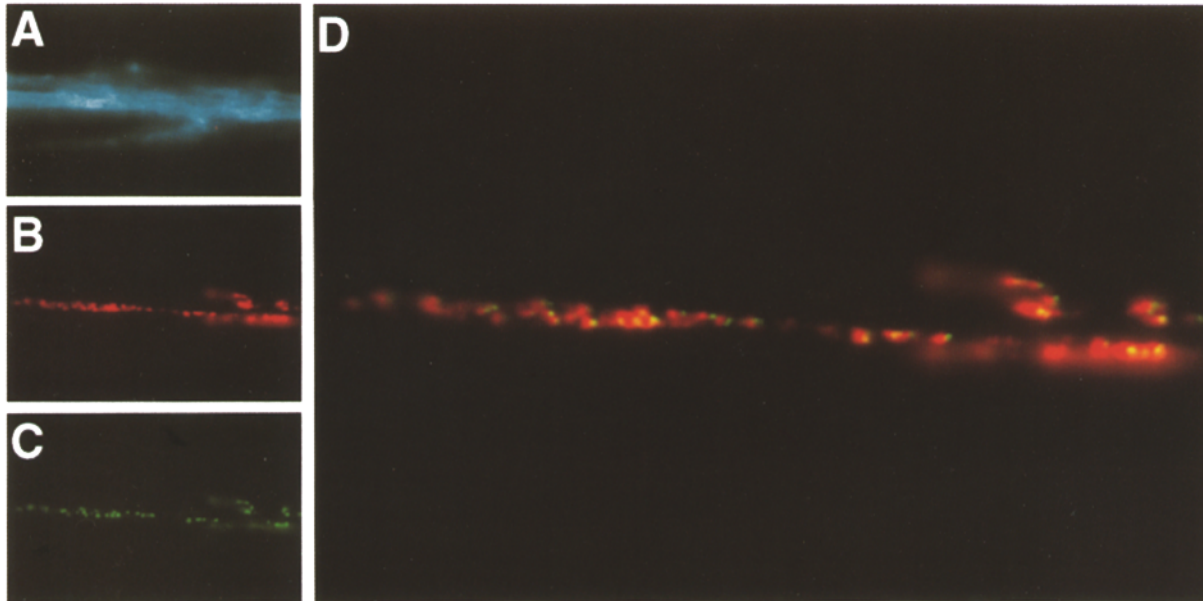
tinomycin D (Fig. 8 F) and 1  $\mu\text{g/ml}$  of  $\alpha$ -amanitin, strongly suggesting that it was driven by pol II (data not shown).

#### **rRNA Synthesis and Processing Domains**

To evaluate the association of rRNA synthesis and process-

ing, we localized sites of pol I transcription relative to sites rich in fibrillar. As noted above, rRNA synthesis was detected in spherical bodies that appeared to be connected and formed necklace-like structures in the majority of unspread nucleoli (Fig. 7 C). The spherical bodies, whether in isolated nuclei or in spread nuclei, were embedded in areas that





**Figure 9** rRNA synthesis and processing domains in the nucleolus. Spread nuclei were prepared as described in Fig. 1, except that the incubation in 1 M Tris-PO<sub>4</sub> (pH 9.1) was done for 2 min to extensively spread the nucleoli. The spread nuclei were washed in transcription buffer and incubated for 1 min at 37°C in this buffer supplemented with NTPs (UTP was replaced with BrU). Spread nuclei were washed and fixed with 2% paraformaldehyde and stained with antisera or antibodies and with Hoechst dye. (A) Spread nucleus stained with Hoechst. (B) Same spread nucleus stained with anti-fibrillarin serum and a secondary anti-human IgG coupled to rhodamine. (C) Same spread nucleus stained with anti-BrU monoclonal antibody and a secondary anti-murine IgG coupled to fluorescein. (D) Same spread nucleus showing overlap (yellow) of staining for BrU (rRNA synthesis) and fibrillarin (rRNA processing).

stained strongly for fibrillarin (Fig. 9 and data not shown). The relevance of the colocalization of nascent rRNA and fibrillarin was tested by increasing the degree of nucleolar spreading. In minimally elongated nucleoli, the spherical bodies remained associated in necklace-like structures (Fig. 8, B and D). In slightly more elongated nucleoli, the spherical bodies formed linear arrays, which appeared to be unfolded necklaces (Fig. 7 G). In highly elongated nucleoli, the spherical bodies, which contained the nascent rRNA, were separated from each other (Fig. 9 B). In these cases, areas staining for fibrillarin surrounded and overlapped sites of rRNA synthesis (Fig. 9, C and D). The coincidence of nascent transcripts with fibrillarin in these highly spread nucleoli suggested very strong interactions between synthetic factors and elements of the processing machinery.

## Discussion

### Nuclear Spreads: A Tool to Probe Structure and Function

Extensive spreading of chromatin is required to determine if RNA synthesis and processing components are structur-

ally linked or simply near neighbors by default in a crowded nucleus. Spread nuclei separate nuclear components over hundreds of microns. Despite dramatic alterations in nuclear morphology, spread nuclei retained pol I in a functional state and associated with other nucleolar components. We have shown that rRNA synthesis in the spread nuclei is in all ways tested like synthesis in intact nuclei. Because of the great dilution of soluble components in the spreading procedure, we suggest that associations imply functional relationships. Thus, as the chromatin is spread over hundreds of microns, as opposed to the few microns in a whole nucleus, noninteracting but neighboring components should be separated, whereas structurally linked components should remain associated. It is now possible to probe the role of spatial associations among components involved in functions preserved in the spread nuclei.

The majority of the ~4 m of DNA in the CV-1 cell nucleus is organized into nucleosome cables (~30 nm in diameter) and those units must have been only partially uncoiled by the spreading procedure. In a spread nucleus 1 mm in length, one fiber must be assembled from many strands of double-helical DNA. The fact that the nuclear lamins, fibrillarin, and many other proteins are retained in the spreads indicates

**Figure 8.** Actinomycin D inhibits transcription in spread nucleoli. Spread nuclei prepared as described in Fig. 1 were washed in transcription buffer and incubated for 30 min at 37°C in this buffer supplemented with NTPs (UTP was replaced with BrU). HeLa cell nuclear extract was included in the transcription mix. Spread nuclei were washed and fixed with 2% paraformaldehyde and stained with antisera or antibodies and with Hoechst dye. (A) Spread nucleus stained with Hoechst after the transcription reaction. (B) Same spread nucleus stained with anti-BrU monoclonal antibody and a secondary anti-murine IgG antibody coupled to rhodamine. (C) Spread nucleus stained with Hoechst after the transcription reaction. (D) Same spread nucleus stained with anti-BrU monoclonal antibody and a secondary anti-murine IgG antibody coupled to rhodamine. (E) Spread nucleus stained with Hoechst after a transcription supplemented with 10 µg/ml actinomycin D. (F) Same spread nucleus stained with anti-BrU monoclonal antibody and a secondary anti-murine IgG antibody coupled to rhodamine. No, nucleoli. Extranucleolar incorporation of BrU was seen to coincide with Hoechst staining strands of spread nuclei.

that only a subset of associations between nuclear components are disrupted by the spreading procedure. Similar buffers have been used previously to visualize the newly synthesized RNA from *Drosophila* nuclei in Miller spreads, and they were found to leave intact many protein-DNA and protein-RNA complexes (Miller and Beatty, 1969; Mougey et al., 1993). The harshness of the extraction conditions and the great dilution of the cell contents in a flow system ( $10^3$ – $10^4$  cells occupying a volume of 2–20 nl were diluted into 100  $\mu$ l of spreading buffer) would favor the dissociation of weak interactions. Associations that remain should be strong and possibly functionally relevant. The preservation of rRNA and mRNA synthesis demonstrated the functional integrity of the spreads. Moreover, when a nucleus was spread over 1 mm, physical proximity on the scale of 200–400 nm implied a much closer association than in a 5- $\mu$ m diameter nucleus.

### **The Nucleolus: A Superassembly of rRNA Synthesis and Processing Domains**

All nascent rRNAs were found within spherical bodies with diameters of  $\sim 0.5$   $\mu$ m. Similar spherical bodies seemingly connected in a necklace structure have been observed before using antibodies to the pol I transcription factor UBF (Zatsepin et al., 1993). Indirect probing for rDNA genes by detection of Ag-NOR proteins have also revealed the existence of similar bodies (Robert-Fortel et al., 1993). In cells treated with the adenosine analogue DRB, anti-pol I antibodies detect similar but much more dispersed structures (Scheer and Rose, 1984; Scheer et al., 1984; Haaf et al., 1991). These previous reports on spherical bodies in necklace patterns speculated that these bodies were the sites of rRNA synthesis. The recent discovery of incorporation of BrU in discreet nucleolar foci by Jackson et al. (1993), Wansink et al. (1993), and Hozák et al. (1994) suggested that these foci were sites of rRNA synthesis. Our work noting BrU incorporation in a necklace-like pattern has more firmly established the identity of these bodies as the sites of rRNA synthesis.

There should be in the order of 200–400 rRNA genes in diploid CV-1 cells (Sollner-Webb and Mougey, 1991; Lewin, 1980), leading us to speculate that there may be as many as  $\sim 20$  rRNA genes per spherical body. How many of these are active is not clear, however, it is likely to be approximately one fifth (Haaf et al., 1991). Other estimates of how many rRNA genes inhabit one of the spherical bodies vary widely. Based on reasonable but unproven assumptions about the minimum diameter of an active rRNA transcription unit, Scheer et al. (1984) concluded that each bead in a necklace contains one active rRNA gene. Jackson et al. (1993) concluded that each of the BrU containing foci had six active genes.

The fact that rRNA synthesis was always coincident with fibrillar in is consistent with data that supports the cotranscriptional processing of rRNA (Grainger and Maizels, 1980) or cotranscriptional assembly of processing complexes on the emerging rRNA (Mougey et al., 1993). The terminal particle (terminal "ball") on rRNAs contains factors involved in the early steps of rRNA processing (Scheer and Benavente, 1990; Mougey et al., 1993). U3 snRNP and fibrillar in are required for these early processing steps (Kass et al., 1990; Tollervey et al., 1993). The terminal particle

is found in short transcripts, suggesting a very early assembly of processing factors on the nascent rRNA.

A tight physical association between sites of rRNA synthesis and sites of rRNA processing within nucleoli is strongly suggested by our data. This association must be stronger than the cohesion of sites of rRNA synthesis, given that sites of synthesis remained associated with fibrillar in but not with each other after spreading of nucleoli. These findings have led us to postulate the existence of stable bipartite domains, which contain all components required to synthesize and process rRNA. These domains may also be the sites of ribosome formation. The bipartite nucleolar domains, with synthetic and processing functions, may be similar to "transcript domains" in the nucleoplasm (Carter et al., 1993). In the nucleoplasm, pol II domains do not coalesce into a single superassembly, probably because unlike nucleolar domains, each nucleoplasmic domain has a unique constellation RNAs and proteins (Weeks et al., 1993; Zehler et al., 1992). This conservation of architecture would parallel conservation of subunits, some shared, some homologous, among the three nuclear RNA polymerases and conservation of initiation factors such as the TATA binding protein.

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