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Spatio-Temporal Dynamics at rDNA Foci: Global Switching Between DNA Replication and Transcription

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Abstract We have investigated the in situ organization of ribosomal gene (rDNA) transcription and replication in HeLa cells. Fluorescence in situ hybridization (FISH) revealed numerous rDNA foci in the nucleolus. Each rDNA focus corresponds to a higher order chromatin domain containing multiple ribosomal genes. Multi-channel labeling experiments indicated that, in the majority of cells, all the rDNA foci were active in transcription as demonstrated by co-localization with signals to transcription and fibrillarin, a protein involved in ribosomal RNA processing. In some cells, however, a small portion of the rDNA foci did not overlap with signals to transcription and fibrillarin. Labeling for DNA replication revealed that those rDNA foci inactive in transcription were restricted to the S-phase of the cell cycle and were replicated predominantly from mid to late S-phase. Electron microscopic analysis localized the nucleolar transcription, replication, and fibrillarin signals to the dense fibrillar components of the nucleolus and at the borders of the fibrillar centers. We propose that the rDNA foci are the functional units for coordinating replication and transcription of the rRNA genes in space and time. This involves a global switching mechanism, active from mid to late S-phase, for turning off transcription and turning on replication at individual rDNA foci. Once all the rRNA genes at individual foci are replicated, these higher order chromatin domains are reprogrammed for transcription. J. Cell. Biochem. 94: 554–565, 2005.

Key words: cell nucleolus; rRNA genes; ribosomal RNA; DNA replication; rDNA replication; rDNA transcription; immunohistochemistry and fluorescence in situ hybridization; fine structure immunohistochemistry

The RNA and DNA polymerases represent highly efficient enzyme complexes that use the DNA template to synthesize nascent RNA and DNA, respectively. Studies of DNA replication in mammalian cells have demonstrated that replication occurs at discrete sites or foci [Nakamura et al., 1986; Nakayasu and Berezney, 1989]. Each replication site or focus is believed to contain numerous replicons that are arranged in multi-loop chromatin domains with an average size of ~1 Mbp [Jackson and Pombo, 1998; Ma et al., 1998; Berezney, 2002; Dimitrova and Berezney, 2002].

A fundamental question is how the processes of DNA replication and transcription are coordinated within mammalian cells. While it is generally assumed that individual genes during DNA replication are not simultaneously active in transcription, little detailed information is available on this matter. Studies of

Abbreviations used: BrU, bromouridine; BrUTP, 5-bromouridine 5′-triphosphate; DFC, dense fibrillar components; dT, 2′-deoxythymidine; FC, fibrillar centers; rDNA, ribosomal gene; rRNA, ribosomal RNA.

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simultaneous replication and transcription at the 1 Mbp chromatin domains identified in mammalian cells have generally indicated that the two processes do not occur at the same time [Wansink et al., 1994; Wei et al., 1998, 1999; Koberna et al., 1999; see however, Hassan et al., 1994]. These findings suggest that whether or not a gene will replicate or transcribe at a given time may be controlled in part at the level of the higher order chromatin domain in which the gene of interest is positioned [Berezney and Wei, 1998; Berezney, 2002].

In contrast to these results concerning overall replication and transcription of the mammalian genome, no studies to date have examined the spatio-temporal relationships of DNA replication and transcription at the individual gene level. With this in mind, we are investigating the spatio-temporal organization and coordination of replication and transcription of rRNA genes. Several features make these genes an outstanding system to study how highly transcribed gene sequences are regulated with respect to DNA replication and the cell cycle. First of all, a portion of the rRNA genes are very actively transcribed in the cell and this portion is exclusively localized in the interior of the nucleolus, which is apparently devoid of other transcribed DNA sequences.

Approximately 400 copies of rDNAs are present in the human diploid genome at well described positions on five pairs of chromosomes in the form of head to tail tandem repeats [Hadjiolov, 1985; Hernandez-Verdun et al., 2002; Olson et al., 2002]. Up to several dozen discrete rDNA foci can be identified in individual nucleoli suggesting that each focus corresponds to a higher order chromatin domain containing multiple rRNA genes [Haaf and Ward, 1996; Koberna et al., 2002; Raška, 2003; Raška et al., 2004]. Previous studies have also demonstrated discrete sites of transcription in the nucleolus that co-localize with the rDNA foci [Koberna et al., 2002].

Using fluorescence microscopic approaches, we have investigated both replication and transcription at individual rDNA foci. Replication of the rDNA involves a temporal sequence of reprogramming at individual foci from active transcription to active replication. Following replication, the rDNA foci are reprogrammed for transcription. Moreover, rDNA foci active in transcription are characterized by the presence of the protein fibrillarin, while those foci active in replication do not co-localize with this protein. Extension of these studies to the electron microscopic level indicates that both DNA replication and transcription occur within the dense fibrillar components (DFC) region of the nucleolus in close apposition to the fibrillar centers (FC).

MATERIALS AND METHODS

Cell Culture, Synchronization, and Drugs Treatment

HeLa cells were grown either in flasks or on circular 12-mm coverslips in Petri dishes and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Sigma), 1% glutamine, 1% penicillin, 1% streptomycin, and 0.85 g/L $NaHCO_3$ at 37°C in a humidified atmosphere containing 5% CO_2. Frequently, synchronized cells were used. Cell synchronization was achieved at the G_1/S border by a double 2′-deoxythymidine (dT) block. The cells were incubated in DMEM containing 3 mM dT for 16 h, then in a fresh medium for 12 h and again for 16 h in a medium with 3 mM dT. After a 100-min incubation in normal medium, more than 90% of the cell population were synthesizing DNA as judged by immunocytochemistry and FACS analysis [see also Malínský et al., 2001]. When an unsynchronized cell population was used, we limited ourselves to cells showing regular nucleoli by phase contrast, that is, to truly interphase cells. S-phase cells were recognized by the presence of replication signal.

The inhibition of RNA polymerase I transcription was achieved by the addition of actinomycin D (0.04 μg/ml for 3 h) as described by Perry [1963]. The inhibition of extranucleolar transcription was achieved by the addition of α-amanitin (10 μg/ml by means of the hypotonic shift) as described by Koberna et al. [1999].

Incorporation of Marker Nucleotides in Nascent Nucleic Acids into Permeabilized and Living Cells

Two different systems for the labeling of nascent DNA and RNA were used. When nucleotides were incorporated into permeabilized cells, the protocol of Raška et al. [1995] was followed. Cells were washed three times in cold PBS, pH 7.4, then three times in ice-cold buffer D (100 mM KCl, 50 mM Tris-Cl, pH 7.4, 5 mM $MgCl_2$, 0.5 mM EGTA, 1 mM PMSF, 1 mM DTT, 160 mM sucrose, 10% glycerol), permeabilized in buffer D containing 0.2% Triton X-100 for

3 min on ice and washed three times in ice-cold buffer D. Transcription was performed in buffer D supplemented with 25 mM S-adenosyl-L-methionine, 5 U/ml RNase inhibitor from human placenta (Roche), 0.2 mM adenosine 5′-triphosphate, guanosine 5′-triphosphate, cytosine 5′-triphosphate, and 5-bromouridine 5′-triphosphate (BrUTP) for 5 min at 32°C. Transcription was stopped by washing with cold PBS followed by fixation in 2% paraformaldehyde in PBS.

Alternatively, marker nucleotides were incorporated in living cells by means of hypotonic shift. The hypotonic shift was performed for 5 min at 37°C, as described previously [Koberna et al., 1999]. HeLa cells were quickly rinsed in pre-warmed buffer KHB (30 mM KCl, 10 mM HEPES, pH 7.4), then overlaid with buffer KHB containing 20 mM BrUTP (Sigma) and/or 0.2 mM biotin-16-dUTP (Roche), incubated for 5 min in a humidified chamber at 37°C, washed and incubated in DMEM containing 10% FCS in 5% CO_2 at 37°C for 5 min.

rDNA Probe and Antibodies

The digoxygenin-labeled rDNA probe was prepared from a pA construct [Erickson et al., 1981] kindly donated by Prof. James Sylvester (Nemours Children's Clinic Research, Jacksonville, Florida). This construct involves a sequence spanning ~200 nucleotides of the 18S rRNA gene, internal transcribed spacer 1, sequence for 5.8 S rRNA gene, the internal transcribed spacer 2, and ~4,500 nucleotides of the 28 S rRNA gene at the upstream end. The probe was labeled with digoxygenin-11-dUTP by nick translation using a DNA labeling kit (Enzo).

The following antibodies were used: mouse anti-digoxygenin antibody (Roche), rat anti-5-bromo-2′-deoxyuridine antibody (Harlan Sera-Lab), rabbit anti-biotin antibody (Enzo), mouse anti-fibrillarin monoclonal 72B9 antibody [Reimer et al., 1987; kindly provided by Dr. K.M. Pollard, Scripps Research Institute, La Jolla, CA], monospecific human autoimmune serum S4 containing antibodies to fibrillarin kindly provided by Prof. G. Reimer (Department of Dermatology, University of Erlangen-Nurnberg, Federal Republic of Germany).

The secondary anti-rabbit, anti-mouse, and anti-rat antibodies conjugated with FITC, Cy3 and Cy5 were kindly donated by Jackson ImmunoResearch Laboratories. In the electron microscopy experiments, 6 nm (kindly donated by Jackson ImmunoResearch Laboratories) or ultra-small grade anti-mouse gold adduct (Aurion) was used.

In situ Hybridization and Light Microscopy

If not otherwise specified, the cells growing on coverslips were washed in cold PBS and fixed/permeabilized in methanol at −20°C for 30 min, rinsed in acetone for 20 s and air-dried. Before denaturation, the cells were incubated with 100 μg/ml RNAse A (Roche) for 2 h at 37°C, progressively dehydrated in ice-cold 30%, 50%, 70%, 96%, and 100% ethanol and air-dried. The denaturation of the cells was performed in 70% deionized formamide/2× SSC, pH 7.0 at 75°C for 2–3 min. The cells were then placed in ice-cold 70%, ethanol, dehydrated and air-dried. The probe was denatured in deionized formamide at 75°C for 8 min and placed on ice. Six microliters of hybridization mix, containing 25 ng of probe, 0.5 mg/ml sonicated salmon sperm DNA, 2 mg/ml *Escherichia coli* tRNA, 50% deionized formamide, 2× SSC, 0.2% BSA, and 10% dextran sulfate, was used for each coverslip. The hybridization was done overnight at 37°C in a humidified chamber. After the post-hybridization washes, coverslips were incubated with 200 U/ml of RNase H (Sigma) at 37°C for 2 h for the elimination of possible RNA-DNA duplexes.

Before immunolabeling, the cells were washed in PBS and incubated for 10 min with 0.5% BSA in order to block non-specific binding. Subsequently, the cells were incubated with primary and secondary antibodies for 60 min at room temperature. The combined detection of fibrillarin as well as incorporated biotin-16-dUTP and BrUTP and in situ hybridization was performed as described previously [Haaf and Ward, 1996]. After immunolabeling, the cells were post-fixed overnight with methanol:acetic acid (3:1) at −20°C. Following hybridization as described earlier, specimens were embedded in a ProLong antifade kit (Molecular Probes) and viewed using a Leica TCS NT laser scanning confocal microscope equipped with a 60× objective (NA = 1.4) and an Krypton-Argon laser with emissions at 488/568/647 nm. Optical sections (0.5 μm), 120-nm apart, were collected. Image restoration was performed using the maximum likelihood estimation algorithm (Huygens 2 system, Scientific Volume Imaging BV). In some experiments, the specimens were

viewed using an Olympus Provis microscope. Images were captured by a charge-coupled device camera (PXL with KAF 1400 chip; Photometrics) running on IPLab Spectrum software (Scanalytics). We avoided multinuclear cells as well as cells with large nuclei possessing apparently highly elevated genome copies, which were occasionally seen in the culture. Location of nucleoli was verified by phase-contrast microscopy.

Electron Microscopy Detection of Incorporated Nucleotides and Fibrillarin

The ultrastructural mapping of newly synthesized DNA as well as rDNA hybridization signal was achieved using a pre-embedding approach [Griffiths, 1993]. The cells with biotinylated nucleotides incorporated into nascent DNA were fixed in 2% PFA in PBS for 10 min, washed in PBS, incubated for 2 min in each 30%, 50%, 70%, 90%, 70%, 50%, and 30% ice-cold methanol, and washed in PBS. Prior to immunolabeling, the cells were treated with 0.05 M glycine in PBS and then with 0.5% BSA in PBS to block non-specific binding. Biotin was visualized with an anti-biotin antibody followed by an anti-mouse antibody ultrasmall gold adduct. Silver intensification was performed according to Danscher [1981]. Finally, the cells were post-fixed in 8% formaldehyde, and dehydrated in gradually increasing ethanol concentrations and propylenoxide, and embedded in epon (Merck).

Fibrillarin was detected on cryosections as described previously [Raška et al., 1995]. HeLa cells were fixed in 4% paraformaldehyde in 0.2 M PIPES, pH 7.0 at 4°C for 2 h, rinsed in a cold PBS, scraped and pelleted at low speed. Pelleted cells were infused with a mixture of sucrose and polyvinylpyrrolidone according to Tokuyasu [1989] and frozen. Thin cryosections were cut with a Drukker diamond cryoknife (Drukker) mounted on a Reichert Ultracut S ultramicrotome (Leica) equipped with a cryoattachment.

BrU-labeled RNA was detected on lowicryl sections. HeLa cells with incorporated BrU were fixed in 8% PFA in 0.2 M Pipes, pH 6.95 for 12 h, washed in PBS, dehydrated in ethanol, and embedded in lowicryl K4M at low temperature [Raška et al., 1995].

Ultrathin epon and lowicryl sections were cut on a Reichert Ultracut E microtome equipped with a Drukker diamond knife (Drukker). The lowicryl sections on electron microscope grids were incubated with anti-bromodeoxyuridine antibodies, washed in PBS and incubated with 6-nm gold anti-mouse adduct. All sections were stained with uranyl acetate and viewed using a Zeiss EM 900 electron microscope.

RESULTS

Mapping rRNA Synthesis With Fluorescence Microscopy

We have mapped nucleolar transcription both in permeabilized cells and in cells that incorporated marker nucleotide into RNA in vivo. The former approach represents a standard method [Dundr and Raška, 1993; Jackson et al., 1993; Wansink et al., 1993], in which nascent rRNAs stay at the site of transcription after the completion of synthesis [Staněk et al., 2000]. The latter approach, implementing a hypotonic shift, was previously shown to represent a relatively gentle and fully reversible procedure for handling cells [Koberna et al., 1999; Staněk et al., 2001]. In agreement with a previously published study [Koberna et al., 2002], both approaches yielded a similar nucleolar transcription pattern, consisting of numerous (10–40) fluorescence foci per cell nucleus. Due to the limited resolution of light microscopy, some spatially close foci appeared to be fused (Fig. 1B,J; see also Fig. 2B). Selective inhibition of nucleolar transcription, by low concentrations of actinomycin D, and its insensitivity to alpha-amanitin, in concentrations sufficient for inhibition of the extranucleolar transcription signal, supported precursor-rRNA synthesis in these foci [data not shown; Koberna et al., 1999; Staněk et al., 2000].

Co-Localization of Nascent RNA and Fibrillarin With rDNA Foci

Similar to the pattern of nucleolar transcription sites, ribosomal genes were localized in multiple foci (10–40 per cell nuclei; Fig. 1A,F,I,M,R,V). Double labeling of rDNA coding sequences and nascent transcripts revealed that the bulk of the nucleolar transcription signal is associated with rDNA foci (Fig. 1A,B) where each rDNA focus may include ~4–15 active genes [Koberna et al., 2002]. At the same time, a fraction of rDNA foci observed in some cells does not overlap with a transcription signal (Fig. 1A,B,D).

While incorporated BrUTP signal corresponds to transcription sites, the abundant

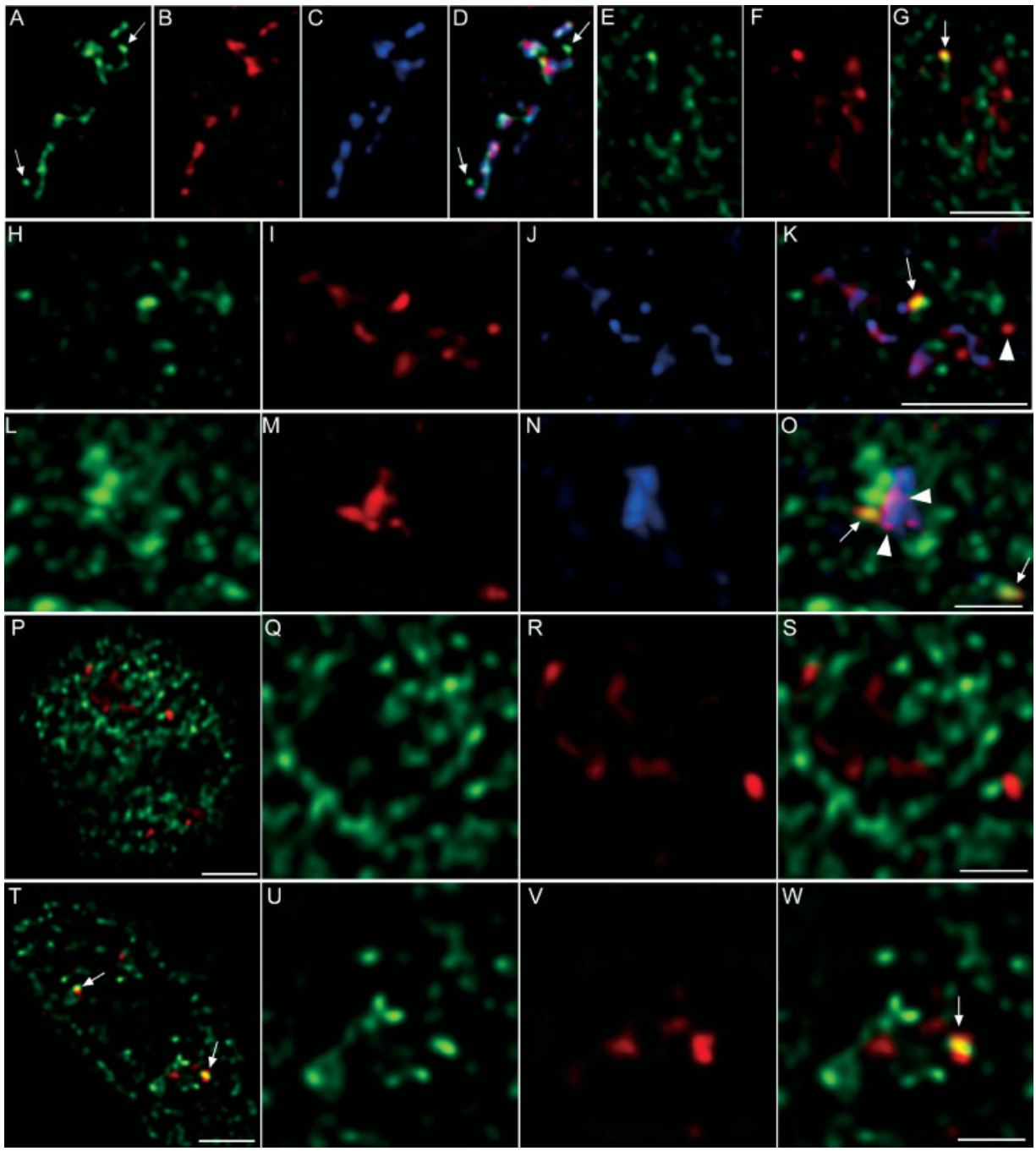

Fig. 1. Laser scanning confocal microscopy of rDNA, nucleolar transcription, replication, and fibrillarin in HeLa cells. Labeling of DNA replication and transcription sites was performed in vivo following hypotonic shift (see Materials and Methods). **A–D**: Fluorescence mapping of rDNAs (A, green), transcription (B, red), and fibrillarin (C, blue); (D) merged image. Most rDNA foci co-localize with transcription signal and fibrillarin. Only two rDNA foci (arrows) are seen outside of transcription/fibrillarin signals. **E–G**: Fluorescence signal of replication (E, green) and rDNAs (F, red). Only one rDNA focus (arrow) clearly exhibits replication activity in the merged image (G). **H–K**: Fluorescence mapping of replication (H, green), rDNAs (I, red), and transcription (J, blue) in the nucleolus. Note that the rDNA focus, which co-localizes with replication signal (arrow), does not contain newly synthesized RNA in the merged image (K). While the majority of rDNA foci are transcriptionally active, foci inactive in both DNA replication and transcription are occasionally observed (see arrowhead in K). **L–O**: Fluorescence mapping of replication (L, green), rDNAs (M, red), and fibrillarin (N, blue) in the nucleolus. In the merged image (O), no fibrillarin signal is seen in replicating rDNA foci (see arrows) while magenta colored sites (see arrowheads) correspond to foci not replicating DNA that co-localize with fibrillarin. **P–S**: Fluorescence mapping of replication sites (Q, green) and rDNA foci (R, red) in early S-phase synchronized cells. No significant overlap was detected in the merged images at lower (P) or higher (S) magnification. **T–W**: Fluorescence mapping of replication sites (U, green) and rDNA foci (V, red) in cells synchronized in mid-to-late S-phase. Co-localization of DNA replication with rDNA foci is detected at two sites in (T) (yellow sites, see arrows) and at higher magnification in (W, see arrow) at one of these sites. All images are 0.5 μm optical sections. Bars: (G, K, P, T) 5 μm; (O, S, W) 2 μm.

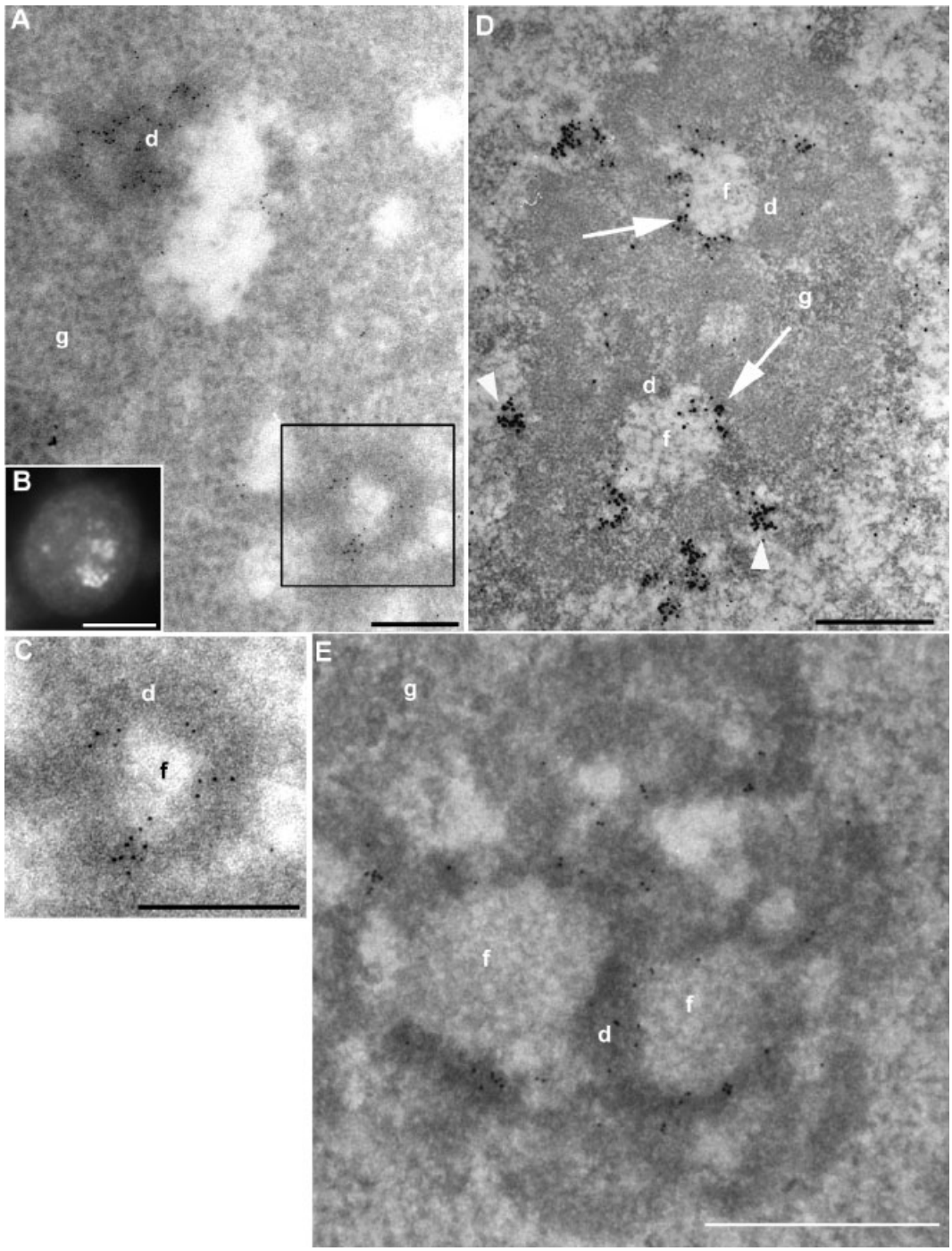

Fig. 2. Electron microscopic localization of replication, transcription, and fibrillarin in HeLa cells. **A**: Electron microscopic localization of transcription in thin sectioned permeabilized cells. **B**: Fluorescence microscopy image of nucleolar transcription pattern seen in permeabilized cells synchronized in S-phase. **C**: Enlarged outlined area in (A). Nucleolar transcription signal consists of numerous clusters of gold particles accumulated in the DFC. **D**: Ultrastructural localization of nucleolar DNA replication reveals that the silver-enhanced immunogold signal is found mainly in the DFC regions (arrows). Note the DNA replication occurring in the perinucleolar chromatin (arrowheads). **E**: EM localization of fibrillarin in a cryosectioned cell reveals the accumulation of gold particles in the DFC. **f**, **d**, **g**: They correspond to fibrillar centers (FC), dense fibrillar components (DFC), and granular components, respectively. Bars: 0.5 μm (A); 10 μm (B); 0.5 μm (C); 1 μm (D); 0.5 μm (E).

nucleolar protein fibrillarin is known to participate in the pre-rRNA processing [Raška, 2003]. Confocal microscopy demonstrated that the fibrillarin signal in the nucleolus is concentrated in numerous foci in sizes similar or somewhat larger that rDNA/transcription foci (Fig. 1A–D). In order to observe localization of this protein with respect to active rRNA, we performed triple labeling of rDNA coding sequences, nascent transcripts and fibrillarin (Fig. 1A–D). Our data demonstrates that transcriptionally active rDNA foci are associated with fibrillarin, which is in agreement with previous studies [Jansen et al., 1991; Cmarko et al., 2000; Staněk et al., 2001]. Moreover, those few rDNA foci that did not co-localize with the transcription signal were devoid of the fibrillarin signal (Fig. 1A–D).

Replication and Transcription Activities Within Individual rDNA Foci Are Mutually Exclusive

In order to map nascent DNA, biotin-16-dUTP was introduced hypotonically into the cultured cells [Koberna et al., 1999]. The fluorescence replication signal was organized in the patterns typical of either early, mid, or late S-phase [Nakayasu and Berezney, 1989; Dimitrova and Berezney, 2002]. No association of nucleolar rDNA foci with replication sites was observed in cells showing early S-phase replication patterns, while in cells exhibiting mid-to-late S-phase replication patterns, a co-localization of a few rDNA foci with replication signal was regularly observed (Fig. 1E–G). Using synchronized cells, we confirmed the absence of detectable replication signal co-localizing with rDNA foci in early S-phase (Fig. 1P–S) and its presence during mid-to-late S-phase (Fig. 1T–W), 5–8 h after S-phase initiation. The majority of the intranucleolar replication sites were not associated with rDNA foci (Fig. 1E–G). In this regard, clumps of condensed chromatin, including newly replicated chromatin, have been identified in nucleoli by means of thin sectioning electron microscopy [Thiry et al., 1991; Raška et al., 1990, 1992, 1995] and rDNAs represent only a small fraction of the total intranucleolar DNA in mammalian cells [Bachellerie et al., 1977]. Triple labeling experiments were then performed to directly investigate the activity of DNA replication (biotin-dUTP) and transcription (BrUTP) at individual rDNA foci in cells synchronized in mid-to-late S-phase. A large proportion of rDNA foci were active in transcription, whereas only a small proportion of foci were typically active in replication (Fig. 1H–K). Co-localization of DNA replication and transcription at individual rDNA foci was never observed (Fig. 1H–K).

In a parallel series of experiments, we simultaneously labeled rDNAs, DNA replication signal, and fibrillarin. Consistently, all transcriptionally active rDNA foci co-localized with this protein, while replicating rDNA foci did not contain signal to the fibrillarin (Fig. 1L–O). This suggests either the displacement of fibrillarin from the replicating rDNA foci or its modification to a form no longer recognized by the fibrillarin antibody. Nucleolar rDNA foci exhibiting neither nascent RNA/fibrillarin nor DNA replication signal were occasionally observed in a small proportion of the rDNA foci (Fig. 1K, arrowhead). These results pointed to the incompatibility of the concomitant transcription/processing activities and DNA replication at the level of individual rDNA foci and indicated that the great majority of rDNA foci not involved in replication are transcriptionally active.

Electron Microscopic Mapping of Nucleolar Transcription, DNA Replication, and Fibrillarin

We next investigated the localization of nucleolar transcription, DNA replication, and fibrillarin at the electron microscopic level using immunogold labeling. As shown in Figure 2A,C, the immunogold labeling of transcription was confined to the DFC of the nucleoli (Fig. 2A,C).

The ultrastructural localization of DNA replication signal in synchronized cells supported and expanded the light microscopy findings. The replication signal, frequently in the form of clusters, was usually found within nucleoli of mid-to-late S-phase cells (Fig. 2D). Apart from replicating clumps of heterochromatin, the signal was regularly confined to the DFC and its border areas with FC, that is, to the same nucleolar subcompartment, which accumulates most of the rDNA signal and is involved in pre-rRNA synthesis [data not shown; Raška et al., 1995, 2004; Koberna et al., 2002; Raška, 2003].

Ultrastructural mapping of fibrillarin was consistent with co-localization of this protein with transcription at the light microscopic level. In agreement with previous studies [Ochs et al., 1985; Raška et al., 1989], fibrillarin was

found enriched in the DFC (Fig. 2E). Only a few gold particles were seen in other nucleolar components.

DISCUSSION

The functions of ribosomal RNA synthesis and processing in mammalian cells occur within the specialized nuclear structures termed *nucleoli* [Busch and Smetana, 1970; Hadjiolov, 1985; Hernandez-Verdun et al., 2002; Raška, 2003]. In recent years, significant progress has been made in understanding the substructural organization of the nucleolus in relationship to rDNA transcription [Raška et al., 1995, 2004; Koberna et al., 2002; Raška, 2003]. Several hundred copies of rDNA in the mammalian diploid genome [Hadjiolov, 1985] are arranged into up to several dozen discrete rDNA foci each containing multiple rDNA genes arranged in tandem array [Haaf and Ward, 1996; Koberna et al., 2002; Raška, 2003]. Consistent with the very active state of rDNA gene transcription, immunolocalization studies have revealed that the great majority of the individual rDNA foci are transcriptionally active at any given time [Koberna et al., 2002].

Conflicting data have been published about replication timing of rRNA genes. While some studies suggest that replication occurs predominantly in mid-to-late S-phase [Giacomoni and Finkel, 1972; Junera et al., 1995], others indicate a significant level of replication during early S-phase [Amaldi et al., 1969; Stambrook, 1974; D'Andrea et al., 1983], or throughout S-phase [Balazs and Schildkraut, 1971; Epner et al., 1981; Berger et al., 1997]. While these conflicting results could stem from the fact that different model systems were used, it is important to note that no studies to date have directly examined the replication of individual rDNA foci within the nucleolus and the temporal relationship of the replicating rDNA foci to their transcriptional activity. With this in mind, we have simultaneously labeled nucleolar sites of DNA replication and transcription in HeLa cells and compared these labeled sites with the rDNA foci using fluorescence in situ hybridization (FISH). As previously reported, we find that the great majority of rDNA foci are active in transcription [Koberna et al., 2002]. Indeed, in most of the cells, virtually all of the rDNA foci are transcriptionally active. These cells are either not in S-phase (no BrdU incorporation) or exhibit an early S-phase type replication pattern [Nakayasu and Berezney, 1989; Dimitrova and Berezney, 2002]. The small portion of cells (<20%) that showed a limited number of rDNA foci not active in transcription, exhibited overall nuclear DNA replication patterns characteristic of mid-to-late S-phase. Moreover, the majority of those rDNA foci inactive for transcription are active in replication.

These findings are consistent with those of Dimitrova and Berezney [2002] who demonstrated that labeling of DNA replication sites within the nucleolar interior is confined to mid and late S-phase. However, we cannot rule out low levels of DNA replication at rDNA foci in early S-phase that are not detected with immunofluorescence microscopy. It has been proposed, for example, that there are two waves for replication of the rRNA genes in S-phase [Berger et al., 1997]. The first wave in early S-phase is believed to represent the transcriptionally active rRNA genes, while the second wave in mid-to-late S-phase is for the transcriptionally inactive genes [Goldman et al., 1984; Berger et al., 1997]. Our findings are not incompatible with this view if only a small portion of rRNA genes at each rDNA focus are transcriptionally active. Further studies are needed to test this possibility.

The temporal relationship between inactive sites of rRNA gene transcription and active replication was further supported by cell synchronization experiments. In cells from early S-phase, virtually all of the rDNA foci were transcriptionally active with no sites of replication detected. In contrast, in mid-to-late S-phase cells, the great majority of cells showed one or more rDNA foci inactive in transcription and active in DNA replication. Triple labeling of DNA replication, transcription, and rDNA foci revealed that the great majority of rDNA that are not active in transcription are active in DNA replication. In no case did we observe rDNA foci active in transcription that were simultaneously active in DNA replication.

This leads us to propose a switching model for the coordinate replication and transcription of rDNA genes in the nucleolus. As outlined in Figure 3, it is proposed that rDNA foci are typically transcriptionally active. Replication of the rDNA genes at individual foci occurs in a progressive fashion during mid-to-late S-phase, but only at foci that are transcriptionally inactive. This suggests the existence of a switching mechanism to turn-off transcription at

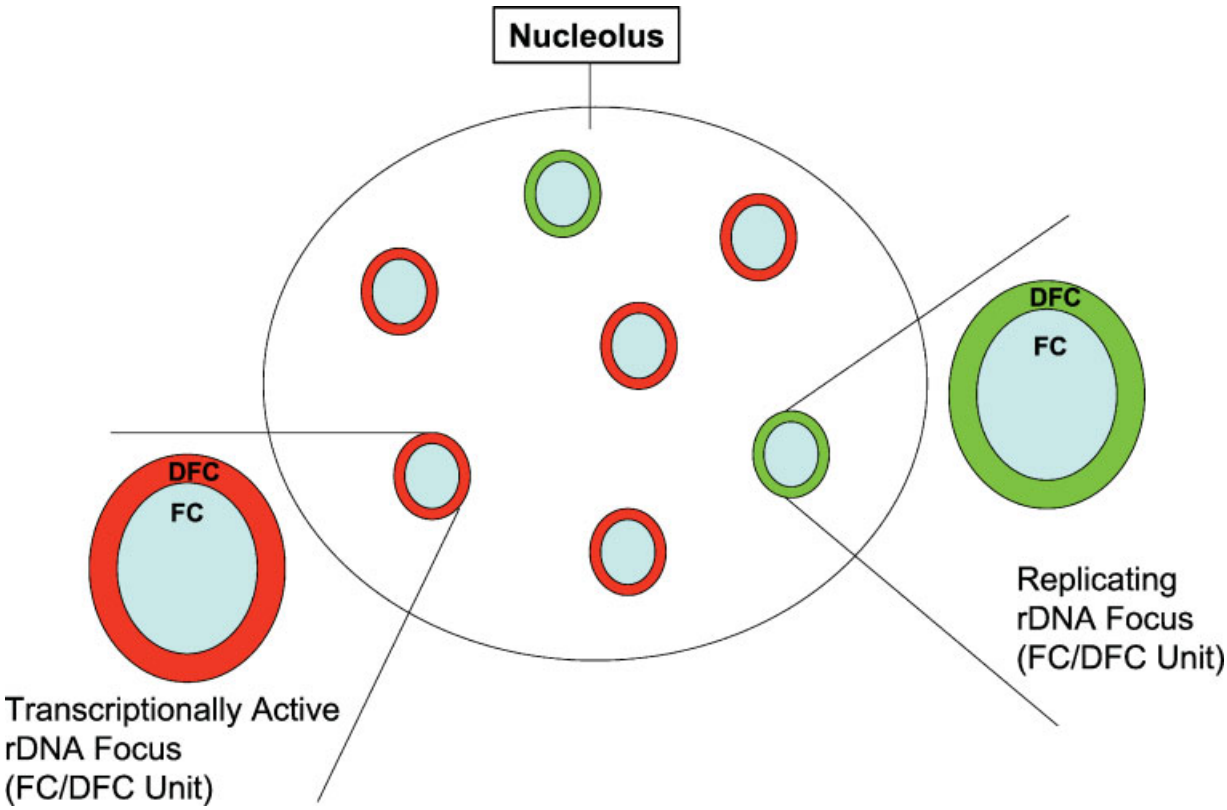

Fig. 3. Schematical representation of rDNA foci dynamically switched ''on'' and ''off'' for DNA replication or transcription. In this model, individual rDNA foci are depicted as being along the borders of the nucleolar FC/DFC regions. Each focus is a functional unit containing multiple rDNA genes switched on for either DNA replication (green) or transcription (red). Following the completion of DNA replication at individual foci, transcriptional activity is switched back on.

these higher order chromatin domains and turn on DNA replication. Following completion of DNA replication, transcriptional activity is switched back on. It is conceivable that there is a short transition period in which some sites are inactive for both replication and transcription. This could explain the very small number of sites that show neither DNA replication nor transcription labeling (see e.g., Fig. 1K, arrowhead).

A similar model has been proposed for the overall coordination of DNA replication and transcription of the genome in the mammalian cell [Berezney and Wei, 1998; Wei et al., 1998; Berezney, 2002]. Our findings provide the first evidence supporting this model at the individual gene level. In further support of this switching model, Scott et al. [1997] reported a progressive decrease in transcriptionally active rDNA from the beginning of S-phase (~90% active transcription) to about 60% in mid S-phase followed by a recovery in late S-phase to levels similar to the beginning of S-phase. We suggest that the progressive decrease in transcriptionally active rDNA is due to a corresponding switching off of transcription at individual rDNA foci activated for DNA replication. The progressive increase of active transcription during late S-phase may be a result of a corresponding switching on of rRNA genes following the completion of DNA replication at individual foci.

While the molecular factors involved in this proposed switching mechanism remain to be identified, our analysis demonstrates that fibrillarin, a characteristic protein of rRNA gene transcription and RNA processing sites [Ochs et al., 1985; Bousquet-Antonelli et al., 1997], is specifically identified at rDNA foci active in transcription but not at those foci active in replication. This may be indicative of a major remodeling of the rDNA foci as the rRNA genes that they contain switch between active states of transcription and DNA replication.

This study also contributes to our understanding of rRNA functional organization at the electron microscopic level. There has been a long-standing debate about the location of active ribosomal genes within the nucleolus [Raška et al., 1995]. At the electron microscopy level, a typical metazoan nucleolus reveals three major components: FC, DFC, and granular components [Busch and Smetana, 1970; Raška et al., 1990, 1992, 2004; Shaw and Jordan, 1995; Olson et al., 2000; Raška, 2003]. Several studies have localized active rRNA genes to the FC [Puvion-Dutilleul et al., 1991; Thiry and Thiry-Blaise, 1991; Thiry et al., 1991, 2000; Scheer et al., 1997; Mais and Scheer, 2001, Cheutin et al., 2002], while other studies demonstrate localization at the DFC together with the DFC/FC border zones [Granboulan and Granboulan, 1965; Wachtler et al., 1989, 1992; Dundr and Raška, 1993; Jimenez-Garcia et al., 1993; Hozák et al., 1994; Raška et al., 1995; Melčák et al., 1996; Mosgöller et al., 1998; Koberna et al., 1999; Koberna et al., 2002; Bassy et al., 2000; Cmarko et al., 2000; Staněk et al., 2000]. Our electron microscopic data demonstrate location of actively transcribed RNA and fibrillarin at the DFC in close apposition to the FC border zones. Moreover, for the first time, DNA replication has been preferentially localized at these same regions of the DFC and the FC border zones. These findings are consistent with the recent results of Koberna et al. [2002] who proposed that the rDNA foci observed with fluorescence microscopy correspond to higher order domains of rDNA genes organized into functional units composed of the FC and surrounding DFC. This is schematically depicted in the model presented in Figure 3.

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