DNA replication initiation patterns and spatial dynamics of the human ribosomal RNA gene loci

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Summary

Typically, only a fraction of the ≥600 ribosomal RNA (rRNA) gene copies in human cells are transcriptionally active. Expressed rRNA genes coalesce in specialized nuclear compartments – the nucleoli – and are believed to replicate during the first half of S phase. Paradoxically, attempts to visualize replicating rDNA during early S phase have failed. Here, I show that, in human (HeLa) cells, early-replicating rDNA is detectable at the nucleolar periphery and, more rarely, even outside nucleoli. Early-replicated rDNA relocates to the nucleolar interior and reassociates with the transcription factor UBF, implying that it predominantly represents expressed rDNA units. Contrary to the established model for active gene loci, replication initiates randomly throughout the early-replicating rDNA. By contrast, mostly silent rDNA copies replicate inside the nucleoli during mid and late S phase. At this stage, replication origins are fired preferentially within the non-transcribed intergenic spacers (NTSs), and ongoing rDNA transcription is required to maintain this specific initiation pattern. I propose that the unexpected spatial dynamics of the early-replicating rDNA repeats serve to ensure streamlined efficient replication of the most heavily transcribed genomic loci while simultaneously reducing the risk of chromosome breaks and rDNA hyper-recombination.

Key words: Gene expression, Human rRNA genes, Nucleolus, Replication factory, Replication origin

Introduction

The nucleolus is the most conspicuous nuclear compartment where several steps in ribosome biogenesis take place: transcription of the 5.8S, 18S and 28S rRNA genes, processing of the 47S pre-rRNA and assembly of pre-ribosomal particles (Hadjiolov, 1985). There are ≥300 copies of rRNA genes in the human genome (McConkey and Hopkins, 1964; Jeanteur and Attardi, 1969; Stults et al., 2008) located in the nucleolar organizer regions (NORs) on the short arms of five pairs of acrocentric chromosomes (Henderson et al., 1972; Hadjiolov, 1985). The number of rDNA repeats in individual human NORs is in the range of 1 to ≥140 (Stults et al., 2008). The levels of rRNA gene transcription vary in different cell types depending on cell proliferation rates and the demand for rRNA. Short-term regulation of rRNA production occurs predominantly through modulation of rDNA transcription rates, whereas long-term regulation also involves changes in the number of transcribed rRNA gene copies (Schwarzacher and Wachtler, 1983; McStay and Grummt, 2008). Only 20–50% of all rRNA genes are transcriptionally active in most human cells (Miller and Bakken, 1972; Gagnon-Kugler et al., 2009). They are believed to exist in a nucleosome-free state, whereas the silent copies are packed as regular chromatin (Hamkalo and Miller, 1973; Sogo and Thoma, 2004). Although the precise organization of active and repressed ribosomal genes within human NORs is unknown, it is clear that some NORs are composed entirely of inactive rRNA genes (silent NORs), whereas other NORs (competent NORs) host active only, or a mixture of active and inactive, rRNA genes (de Capoa et al., 1988). The number of competent NORs varies among cell types and even within the same cell type in each individual; typically, more than half (50–100%) of all NORs in rapidly growing cells are competent (Mikelsaar et al., 1977; Miller et al., 1977; Mikelsaar and Schwarzacher, 1978; Schmiady et al., 1979; Zakharov et al., 1982; Heliot et al., 2000; Kalmarova et al., 2007). During interphase, all competent NORs congregate within one, or a few, nucleoli per nucleus (Anastassova-Kristeva, 1977; Schwarzacher and Wachtler, 1983; Hadjiolov, 1985). Even though the organization and integrity of nucleoli depend on ongoing rRNA synthesis, processing and transport (Hernandez-Verdun et al., 2002), localization to the nucleolar compartment(s) is not restricted to transcriptionally active loci. Thus, many non-rDNA loci (van Koningsbruggen et al., 2010; Puvion-Dutilleul et al., 1991; Nemeth et al., 2010), as well as some, or all, silent NORs (D.S.D., unpublished) (Sullivan et al., 2001; Kalmarova et al., 2007) also associate with nucleoli.

Despite the fact that the discovery of the nucleolus dates back to the late 18th century and early 19th century (Schwarzacher and Wachtler, 1983; Mosgoeller, 2004), and the thousands of publications resulting from many decades of intensive research, consensus about its structural and functional organization has not been reached (Olson, 2004). Although not as hotly debated as the location of rRNA gene transcription, rDNA replication is also associated with unresolved issues regarding both the location of replicating ribosomal genes and the usage of specific replication origins. Attempts to map replication origin positions in the human rDNA loci have generated conflicting results (see the Results and Discussion sections). Furthermore, biochemical studies have revealed that, in mammals, rDNA replicates throughout S phase (Balazs and Schildkraut, 1971; Epner et al., 1981; Berger et al., 1997; Li et al., 2005). It is considered that the transcriptionally active rDNA copies replicate in early S phase, whereas the silent copies replicate in late S phase (Li et al., 2005). However, immunofluorescence microscopy methodologies for visualization of nascent rDNA failed to detect replicative activity inside nucleoli before mid S phase (Junera et al., 1995; Pliss et al., 2005). The

goal of the present work was to investigate the location of the elusive early-replicating rDNA. My findings reveal complex, spatially and temporally differentiated regulation of the replication of active and repressed rRNA genes in the course of S phase, which is possibly relevant to efficient rDNA replication and maintenance of rDNA integrity.

Results

rDNA replication occurs at the periphery of, or outside, nucleoli during early S phase

The expressed rRNA genes are located and transcribed in the nucleolar interior. However, all descriptions of the distribution of DNA replication sites during the first half of S phase state that replication takes place throughout the cell nucleus except inside nucleoli (Nakamura et al., 1986; Nakayasu and Berezney, 1989; van Dierendonck et al., 1989; Banfalvi et al., 1990; Humbert and Usson, 1992; O'Keefe et al., 1992; Hozak et al., 1993; Dimitrova and Berezney, 2002). This is illustrated in Fig. 1 and supplementary material Fig. S1. HeLa cells were incubated for 10 minutes with 5-ethynyluridine (EU) and 5-bromo-2′-deoxyuridine (BrdU) to label nascent RNA and DNA, respectively. The distribution of transcription (EU) foci is similar in all nuclei, whereas replication (BrdU) foci are arranged in different patterns typical for early (~0–5 hours into S phase, Fig. 1A–D), middle (~6–8 hours into S phase, Fig. 1E–G) and late (~9–10 hours into S phase, Fig. 1H) S phase. Nearly a quarter of the total nascent RNA output in HeLa cells is produced by RNA polymerase I (PolI), as measured by nuclear RNA run-on assay (supplementary material Fig. S2), accounting for the strong fluorescence of the nascent rRNA foci. There was no detectable replicative activity inside nucleoli during the first half of S phase, and they appeared as hollow gaps in the early S phase replication pattern (supplementary material Fig. S1A). When transcription and replication sites are visualized simultaneously, these gaps were filled with extremely bright nascent rRNA foci (Fig. 1A–D; supplementary material Fig. S1B). By contrast, several replication foci interspersed among the EU foci were easily detectable in the nucleolar interior throughout mid and late S phase (Fig. 1E–G), with the exception of the final 1–2 hours of S phase (Fig. 1H). These observations raise a puzzling paradox: although light-microscopy experiments fail to detect replication activity inside nucleoli during the first half of S phase (Fig. 1; supplementary material Fig. S1) (Nakamura et al., 1986; Nakayasu and Berezney, 1989; O'Keefe et al., 1992; Junera et al., 1995; Dimitrova and Gilbert, 1999; Pliss et al., 2005), biochemical experiments show that roughly half of all rDNA repeats are replicated at this same time in mammalian cells (Balazs and Schildkraut, 1971; Epner et al., 1981; Berger et al., 1997; Li et al., 2005).

I reasoned that perhaps the solution to this paradox is to look for the early-replicating ribosomal genes not inside, but around the nucleoli. To test this hypothesis, I applied three-dimensional DNA fluorescence in situ hybridization (3D DNA FISH) to detect rDNA in HeLa cells pulse-labeled for 10 minutes with 5-ethynyl-2′-deoxyuridine (EdU) to visualize active replication sites. The relatively small size of the fluorescent azide molecules used for EdU detection allows them to penetrate dense nuclear structures, which might be inaccessible to bulky immunological reagents. For the same reason, fluorescent azide derivatives can react with EdU within double-stranded DNA, eliminating the necessity to use harsh procedures for DNA denaturation and thus ensuring superior chromatin structure preservation – an important advantage for this particular study. To exclude the possibility of artifactual rDNA redistribution caused by heat-induced chromatin spreading or breakage, I also used a gentle DNA FISH methodology, which avoids thermal denaturation of DNA (van Dekken et al., 1988). The rDNA FISH signals appeared as several intense spots of various sizes, interspersed with more diffuse and less intense fluorescent signals (Figs 2–4). To visualize the nucleolar compartment(s), the cells were stained with antibodies specific for the ubiquitous protein nucleolin. Owing to its diverse functions in rRNA production and ribosome assembly (Mongelard and Bouvet, 2007), nucleolin levels are high in nucleoli (Spector et al., 1984; Rickards et al., 2007; Ugrinova et al., 2007), and nucleolin staining is often used as a marker for the nucleolar territories (van Koningsbruggen et al., 2010; Alcalay et al., 2005; Olson and Dundr, 2005; Ma et al., 2007; Amin et al., 2008). Accordingly, all clusters of strongly fluorescent nascent rRNA foci in HeLa cells were embedded within nuclear areas positive for nucleolin immunostaining (supplementary material Fig. S3). Simultaneous immunodetection of the PolI transcription factor, upstream binding factor (UBF) was performed to identify transcriptionally active ribosomal gene copies (Mosgoeller et al., 1998), given that virtually all UBF staining was closely associated with bright EU foci (i.e. nascent rRNA) during interphase in HeLa cells (supplementary material Fig. S4). Consistent with the results of the EU + BrdU labeling experiments (Fig. 1A–H), the three-dimensional confocal analysis of HeLa nuclei revealed that rDNA FISH signals located inside the nucleolin-stained

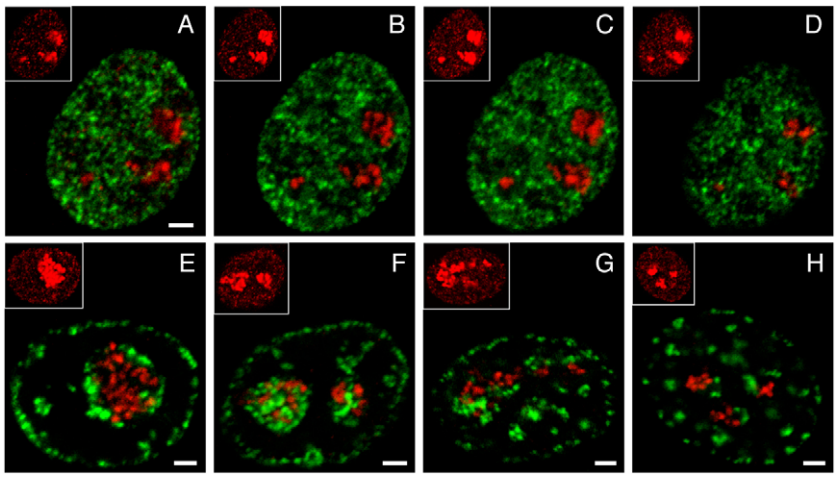

Fig. 1. Spatial organization of PolI transcription foci and DNA replication foci in HeLa cell nuclei during the course of S phase. EU-labeled nascent RNA (red) was stained with Alexa-Fluor-594–azide, and BrdU-labeled nascent DNA (green) was detected with mouse anti-BrdU antibodies plus Alexa-Fluor-488-conjugated anti-mouse-IgG antibodies. In the main images, fluorescence intensity is optimized for visualization of the strong nucleolar EU signals. The insets show brighter images where the nucleoplasmic transcription foci are also visible, however, details in the nucleolar areas are lost. (**A**–**D**) Four consecutive confocal *z*-sections through a single nucleus in early S phase. Replication foci are excluded from the nucleoli (the areas with clusters of bright foci of EU-labeled rRNA). (**E**–**H**) Single confocal *z*-sections through four HeLa nuclei. Replication foci interspersed with transcription foci inside nucleoli are detectable in nuclei in mid S phase (E–G), but rarely in late S phase (H). Scale bars: 2 μm.

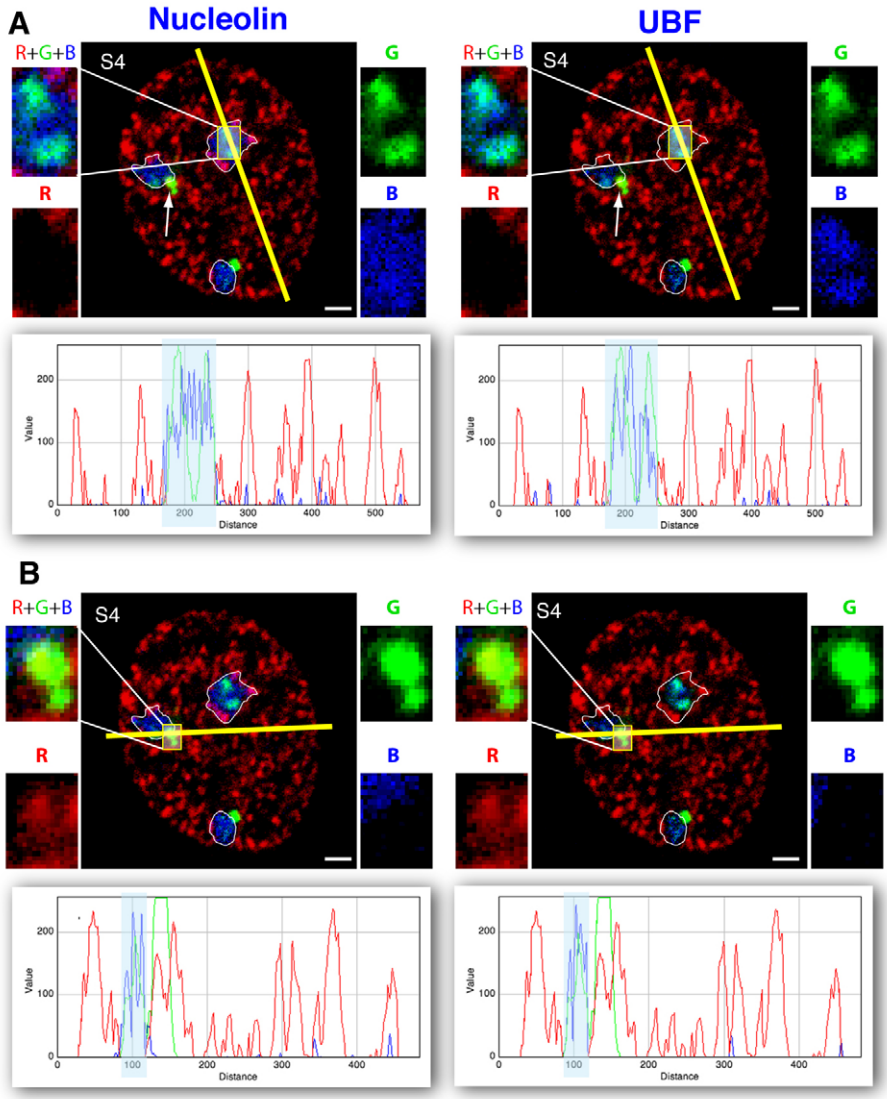

Fig. 2. rRNA genes replicate at the nucleolar periphery during early S phase. HeLa cells were pulse-labeled for 10 minutes with EdU and immediately fixed. 3D DNA immunoFISH was performed to visualize rDNA (green), DNA replicated during the EdU pulse (red), nucleolin (left-hand column, blue) and UBF (right-hand column, blue). A single confocal *z*-section (S4) from a nucleus exhibiting an early S-phase replication pattern is shown. Lines passing through two nucleoli were drawn across the images and the line-scan plots of fluorescence intensity versus distance recorded along the lines are shown below each image. The three-dimensional confocal image stack for this nucleus is shown in supplementary material Fig. S5A. Small areas of interest (framed) are shown at higher magnification alongside the respective image (as RGB, as well as their respective red, green and blue image components). The nuclear areas stained with the anti-nucleolin antibodies (i.e. the nucleoli) are outlined with white contours in the confocal images and are highlighted in a light blue color in the line-scan profiles. rDNA FISH signals are classified as: intranucleolar, when enclosed within the contours; located at the nucleolar periphery, when straddling or touching the contours from the outside; or distant from nucleoli, when separated from the contoured areas by nuclear space negative for nucleolin staining. Replicating rDNA (arrows) does not colocalize with UBF and is detected at the nucleolar periphery (**B**), but not inside nucleoli (**A**) in the early S-phase nucleus. Scale bars: 2 µm.

compartments did not colocalize with EdU in nuclei exhibiting the early S-phase replication pattern (Fig. 2A; supplementary material Fig. S5A; Table 1). Notably, in the majority of early S nuclei, rDNA FISH signals overlapping EdU foci could be found at the periphery of or, more rarely, even outside the nucleolar areas (Fig. 2B, supplementary material Fig. S5A; Table 1). The number of rDNA foci engaged in DNA replication was low at any given moment (one to a few), which could explain why the early-replicating perinucleolar rDNA foci have hitherto eluded detection. Even though both EdU staining (D.S.D., unpublished) and rDNA FISH (D.S.D., unpublished) (Schofer et al., 1998; Pasero et al., 2002; Lebofsky and Bensimon, 2005) are sufficiently sensitive to detect individual extended DNA fibers, it is not possible to exclude that, for unknown reasons, rDNA potentially replicating inside the nucleolar interior during early S phase cannot be detected by light microscopy. However, the positive identification reported here suggests that at least some of the early rDNA replication takes place at the edge of, or outside, nucleoli.

In contrast to early S phase, one or more replicating rDNA domains (i.e. overlapping with EdU) were readily detectable in the nucleolar interior during mid S phase (Fig. 3A; supplementary material Fig. S5B; Table 1), but rarely in late S phase (Fig. 3B; supplementary material Fig. S5C; Table 1), in agreement with

Table 1. Proportions of HeLa cells exhibiting rDNA replication inside, at the periphery, or outside nucleoli at different stages of S phase

S-phase stage	Number of cells analyzed	Cells with rDNA replicating inside nucleoli (%)	Cells with rDNA replicating at the nucleolar periphery (%)	Cells with rDNA replicating outside nucleoli (%)	Cells with no detectable replicating rDNA (%)
Early	128	0 (*n*=0)	53.1 (*n*=68)	12.5 (*n*=16)	34.4 (*n*=44)
Mid	156	41.0 (*n*=64)	43.6 (*n*=68)	12.8 (*n*=20)	2.6 (*n*=4)
Late	52	7.7 (*n*=4)	30.8 (*n*=16)	38.5 (*n*=20)	23.1 (*n*=12)

The scores are from a single experiment. Similar results were obtained in three independent experiments.

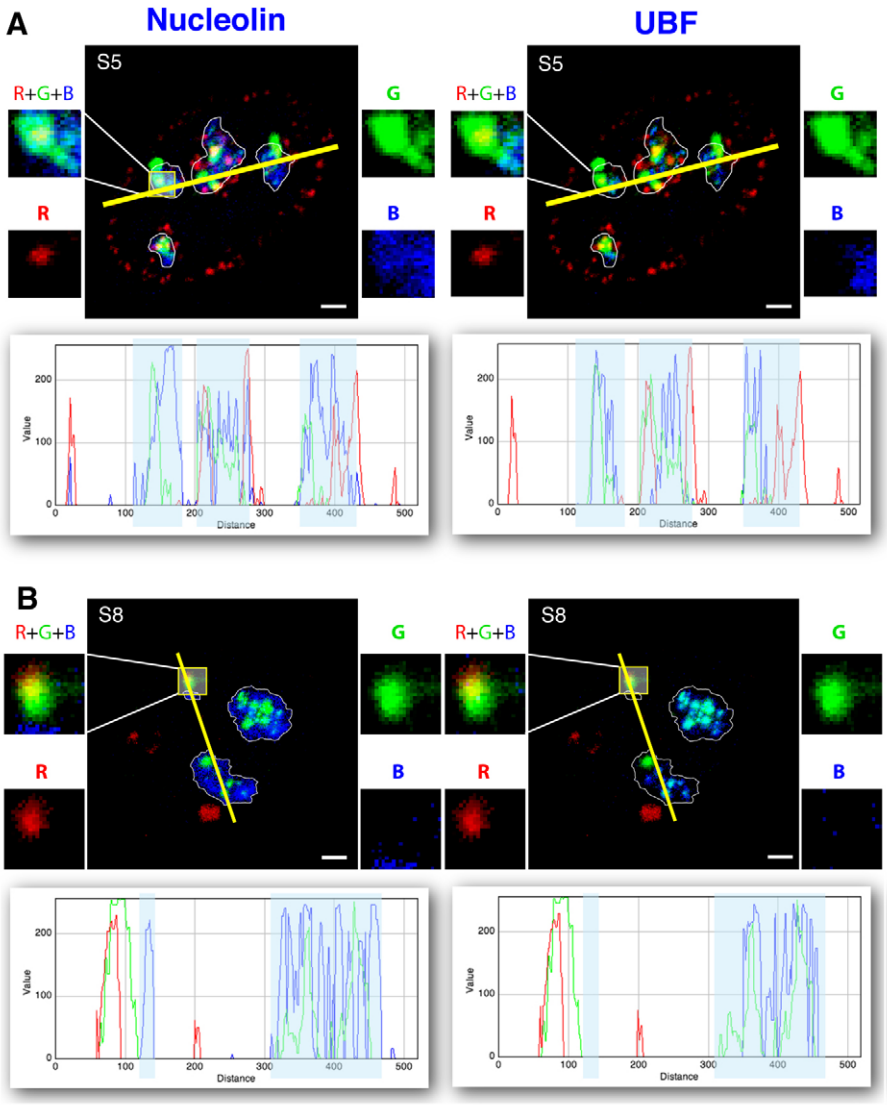

Fig. 3. Location of replicating rDNA during mid and late S phase. HeLa cells were pulse-labeled for 10 minutes with EdU and immediately fixed. 3D DNA immunoFISH was performed to visualize rDNA (green), DNA replicated during the EdU pulse (red), nucleolin (left-hand column, blue) and UBF (right-hand column, blue). Representative single confocal *z*-sections from nuclei exhibiting mid S-phase (**A**) or late S-phase (**B**) replication patterns are shown. Line-scan plots of fluorescence intensity versus distance recorded along the yellow lines drawn across the images are shown below each image. The three-dimensional confocal image stacks for each nucleus are shown in supplementary material Fig. S5B,C. Small areas of interest (framed) are shown at higher magnification alongside the respective image (as RGB, as well as their respective red, green and blue image components). The nuclear areas stained with the anti-nucleolin antibodies (i.e. the nucleoli) are outlined with white contours in the confocal images and are highlighted in a light blue color in the line-scan profiles. Replicating rDNA is located both inside nucleoli and at the nucleolar periphery. Scale bars: 2 µm.

previous reports (Junera et al., 1995; Pliss et al., 2005). In a fraction of nuclei in mid and late S phase, colocalization of rDNA with EdU was also detectable at the nucleolar periphery and outside nucleoli (Table 1). Most intranucleolar replication foci did not contain rDNA (supplementary material Fig. S5B), consistent with the view that only a relatively small proportion of the DNA inside nucleoli represents rDNA (Puvion-Dutilleul et al., 1991).

Altered replication timing in cancer cells has been reported for individual genomic loci (Dotan et al., 2008). Hence, it was possible that, despite the general correlation between active gene expression and early replication timing, as well as the reported early replication of expressed ribosomal genes in immortalized mouse 3T3 fibroblasts (Li et al., 2005), the active rRNA genes might be late-replicating in the transformed HeLa cells. Unfortunately, the UBF staining was not useful for revealing the expression status of the early-replicating peri- or extra-nucleolar, or the late-replicating intranucleolar rDNA units, because they, and replication foci in general, did not overlap significantly with UBF (Figs 2 and 3, UBF). It is possible that the passage of the replication machinery displaces UBF from DNA.

Replicated rDNA relocates to the nucleolar interior and reassociates with UBF

To overcome this difficulty, I performed a pulse-chase experiment to examine UBF reassociation with previously replicated rDNA. Aliquots of HeLa cells pulse-labeled for 10 minutes with EdU (from the same cell population used in Figs 2 and 3) were transferred to EdU-free medium. At hourly intervals thereafter, aliquots of cells were processed for UBF, nucleolin and rDNA immunoFISH combined with EdU detection. The results from a 2-hour chase are shown in Fig. 4 and supplementary material Fig. S6. As shown previously (Manders et al., 1992; Dimitrova and Gilbert, 1999), this time is sufficient for all DNA contained within a replication focus to complete replication. In striking contrast to the ‘real-time snapshots’ during ongoing replication (Fig. 2A,B), EdU-positive rDNA FISH signals were detected inside nucleoli in a large fraction (59.6%, n=146 cells) of the nuclei exhibiting an early-S-phase replication pattern (Fig. 4A, nucleolin; supplementary material Fig. S6A). UBF colocalized with the EdU-tagged rDNA in these cells (Fig. 4A, UBF), implying that the latter predominantly represents expressed rRNA genes. In nuclei exhibiting replication patterns from the second half of S phase, rDNA FISH signals did not show substantial overlap

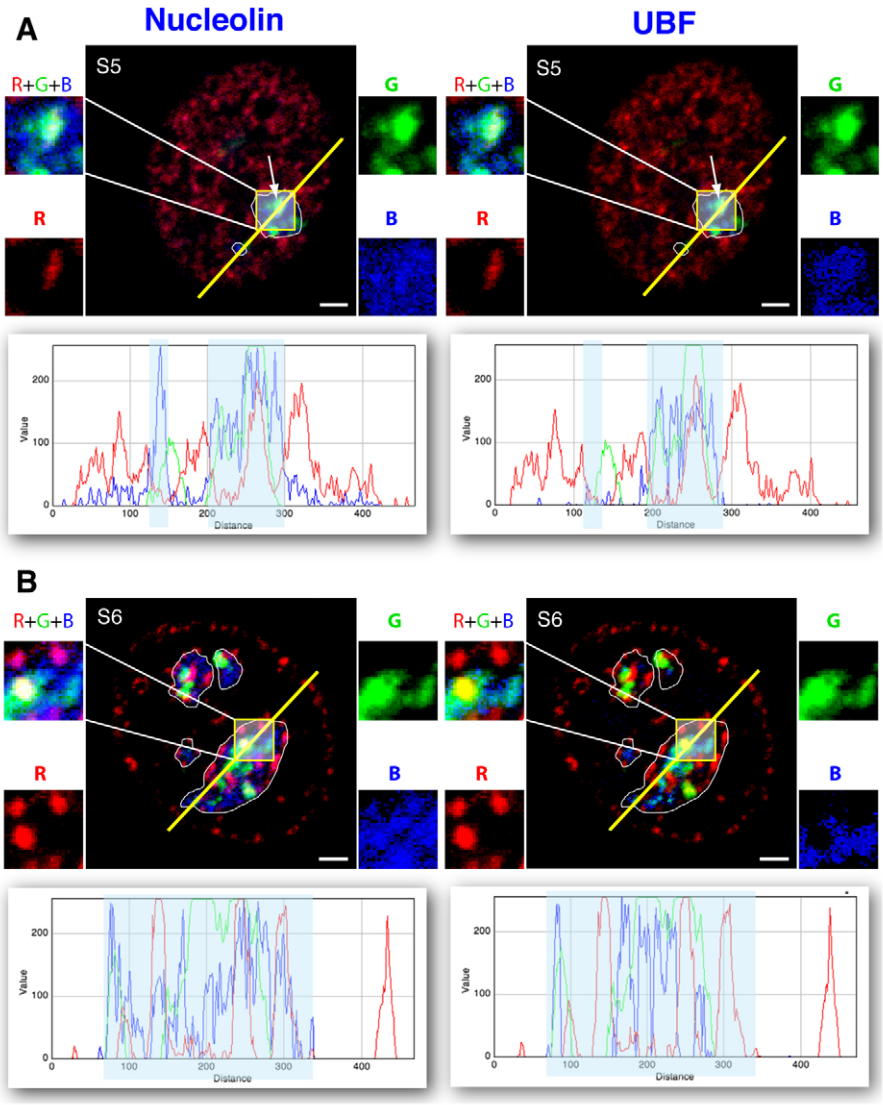

Fig. 4. Inside nucleoli, UBF associates selectively with human rRNA genes replicated during early S phase but not mid S phase. HeLa cells were pulse-labeled with EdU, transferred to free medium and fixed and processed 2 hours later. 3D DNA immunoFISH was performed to visualize rDNA (green), DNA replicated during the EdU pulse (red), nucleolin (left-hand column, blue) and UBF (right-hand column, blue). Representative individual confocal *z*-sections from nuclei exhibiting (**A**) an early S-phase or (**B**) a mid S-phase replication pattern are shown. Arrows point to EdU-overlapping rDNA FISH signals. Line-scan plots of fluorescence intensity versus distance recorded along the yellow lines drawn across the images are shown below each image. The three-dimensional confocal image stacks for each nucleus are shown in supplementary material Fig. S6. Small areas of interest (framed) are shown at higher magnification alongside the respective image (as RGB, as well as their respective red, green and blue image components). The nuclear areas stained with the anti-nucleolin antibodies (i.e. the nucleoli) are outlined with white contours in the confocal images and are highlighted in a light blue color in the line-scan profiles. Scale bars: 2 µm.

with UBF-stained regions after the chase (Fig. 4B, UBF), indicating that, similar to non-transformed cells, the silent rDNA repeats replicate later in S phase in HeLa cells. Similar results were obtained in two more experiments using different populations of HeLa cells. Taken together, these findings suggest that expressed ribosomal genes reposition to the edge of or outside nucleoli before their replication and, once replicated, shift back to the nucleolar interior where they reassociate with UBF and become transcriptionally active. Importantly, the fact that silent rDNA repeats and other DNA are replicated inside nucleoli (Fig. 1E–G; Fig. 3A; supplementary material Fig. S5B) indicates that the densely packed nucleolar interior is not prohibitive for assembly of functional replication factories or for light-microscopy detection of replicating rDNA. Therefore, the surprising spatial dynamics of the expressed ribosomal genes, whereby they engage in replication factories at the nucleolar periphery, might be related to their transcriptional status.

The distribution of replication origin sites in the human rDNA repeats differs from the established model for active gene loci

Replication initiation sites tend to be excluded from transcribed segments of the chromatin fiber (Hyrien et al., 1995; Mesner and Hamlin, 2005; Dimitrova, 2006), and ongoing transcription is essential to sustain this preferential origin positioning (Dimitrova, 2006). Previous studies of the distribution of replication origins in the human rDNA clusters have generated disparate results (summarized in supplementary material Fig. S7) (Little et al., 1993; Yoon et al., 1995; Gencheva et al., 1996; Lebofsky and Bensimon, 2005). The majority of studies (Yoon et al., 1995; Gencheva et al., 1996; Lebofsky and Bensimon, 2005) have made no attempt to look for potential differences between early- and late-replicating ribosomal gene units, whereas those that have addressed this issue (Little et al., 1993) have found no significant differences. To gain further insight into the distinct behavior of expressed and silent rRNA genes, I examined their replication initiation patterns by taking advantage of their different replication timing. Abrogation of the replication checkpoint in cells grown in the presence of aphidicolin (Aph) leads to disassembly of existing replication forks stalled by Aph and progressive activation of later-firing origins in the absence of actual genome replication (Dimitrova and Gilbert, 2000). Importantly, although checkpoint inhibitors prolong S phase, neither the temporal order of replicon activation nor the normal distribution of replication origins are altered (Dimitrova and Gilbert, 2000). Therefore, this approach

can be used to map replication origin locations throughout S phase (see supplementary material Fig. S8 for further details on the methodology). I performed radioactive nuclear DNA run-on with aliquots of HeLa cells synchronized at the G1–S border and either released into S phase in the absence of drugs or maintained in the presence of Aph and the replication checkpoint inhibitor 2-aminopurine (2-AP). ^{32}P-labeled short nascent DNA, prepared at different timepoints, was hybridized to a panel of DNA probes unique to the human rDNA repeats (supplementary material Fig. S7) to reveal the positions of replication forks. As previously reported (Balazs and Schildkraut, 1971), rDNA replication in HeLa cells occurred throughout S phase; however, two peaks in early and middle S phase were detected in the present study (Fig. 5A,B). Analysis of the distribution of replication intermediates in the presence of Aph, which restrains forks close to the replication origins, produced surprising results (Fig. 5A,C). Initiation events were detected at similar frequencies throughout the rDNA unit in cells arrested at the G1–S border or maintained for 3 hours in the presence of Aph + 2-AP. Because this is the time when active ribosomal genes replicate (Figs 2–4), the random initiation pattern is the opposite to what would be expected for the most highly transcribed genomic loci on the basis of the model for the relationship between replication origin location and local transcription (Mesner and Hamlin, 2005). It is unlikely that this is an artifact of the drug treatment, because site-specific replication initiation patterns in other transcribed loci are not altered under the same conditions (Dimitrova and Gilbert, 2000). Similarly, the unexpected result cannot be explained by drug-induced alteration in transcription patterns, because neither chemical affected rDNA transcription rates significantly (supplementary material Fig. S9).

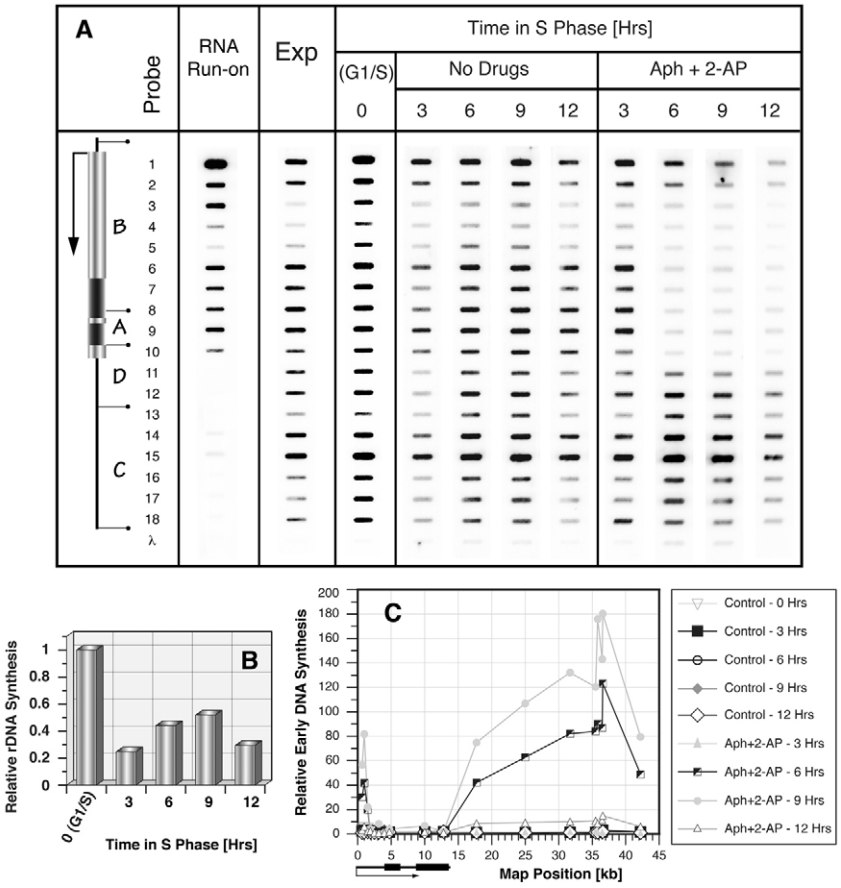

Fig. 5. Replication initiation and elongation patterns in the human rDNA repeats in the course of S phase. (**A**) ^{32}P-labeled nuclear replication run-on products purified from 10^6 HeLa cells synchronized at the G1–S border (0 Hrs) and either released into drug-free medium (No Drugs), or maintained in the presence of Aph + 2-AP for the indicated times, were hybridized to a panel of human rDNA probes (1–18; see supplementary material Fig. S7) and a fragment of phage λ DNA (λ). DNA run-on (Exp) and RNA run-on hybridization results obtained with exponentially growing cells are shown as examples of random distribution of replication forks or to visualize the transcribed portion of the rDNA unit, respectively. The schematic diagram of an rDNA repeat serves to illustrate probe allocation and is not to scale. The horizontal pins on the map mark *Eco*RI sites. The 18S and 28S rRNA coding regions are shown as dark boxes, and the start site and direction of transcription are indicated by the arrow. (**B**) Replication rates in the human rDNA repeats during S phase. In cells released from the G1–S block in the absence of drugs, replication forks move unrestrained and asynchronously, and therefore are positioned randomly throughout the rDNA units. Relative c.p.m. values for all rDNA probes obtained by phosphoimaging of the 'No Drugs, 0–12 Hrs' blots shown in A were summed for each timepoint. Results were normalized to the values obtained for the G1–S-arrested cells and plotted against the elapsed time in S phase. (**C**) Replication initiation frequencies in the human rDNA repeats during S phase. In cells maintained in the presence of Aph + 2-AP replication forks are arrested close to the replication origins. To correct for differences in probe size, base composition and hybridization efficiency, relative c.p.m. values obtained by phosphoimaging of the 'Aph+2-AP, 3–12 Hrs' blots in A were normalized to the corresponding values for exponentially growing cells as described previously (Dimitrova and Gilbert, 1998) and plotted against the map position of each probe. The location of the 47S-rRNA coding region is indicated under the graph.

Equally unexpected and even more striking is the focusing of initiation events to the non-transcribed intergenic spacers (NTSs) in cells maintained for 6–9 hours in the presence of Aph + 2-AP (i.e. the S-phase stage when the silent rRNA genes replicate). Notably, the peak of replication initiation activity coincided with a preferred initiation zone upstream of the rDNA promoter, which had also been identified in some previous studies (supplementary material Fig. S7). These findings provide a potential explanation for the contradictory results generated by earlier rDNA replication origin mapping endeavors (Little et al., 1993; Yoon et al., 1995; Gencheva et al., 1996; Lebofsky and Bensimon, 2005). It is possible that, owing to differences in experimental systems and design, and/or the capabilities of the methodologies involved, individual labs preferentially detected one of the two, or a combination of both, initiation patterns specific for the two classes of ribosomal genes.

RNA PolI-driven transcription is a determinant of the replication initiation pattern in the human rRNA gene clusters

I next asked whether the specific late S-phase initiation pattern is dependent on PolI-mediated transcription. Nuclear DNA run-on was performed with aliquots of HeLa cells synchronized at the G1–S border and released into S phase for 6 or 9 hours in the absence of drugs or maintained in the presence of Aph + 2-AP with or without the addition of 0.1 μg/ml actinomycin D (AMD). AMD treatment results in premature termination of rRNA transcripts. Even though at low doses (0.1 μg/ml), some residual transcription of 5′ external transcribed spacer (5′-ETS) leader sequences persists (supplementary material Fig. S10), as previously described for mouse rDNA transcription (Fetherston et al., 1984; Puvion-Dutilleul et al., 1997), this AMD concentration was selected because it efficiently blocks transcription through most of the rDNA unit but with little or no effect on PolII-directed transcription or DNA replication (Baserga et al., 1965; Fetherston et al., 1984; Dimitrova, 2006). The data presented in Fig. 6 show that selective inhibition of rRNA synthesis resulted in drastic reduction of initiation specificity in the mid and late-replicating rDNA copies, thus uncovering a role for PolI-mediated transcription in setting the replication profile of human rDNA.

Discussion

The lack of replicative activity inside mammalian nucleoli before mid S phase (Nakamura et al., 1986; Nakayasu and Berezney, 1989; O'Keefe et al., 1992; Dimitrova and Berezney, 2002) has puzzled investigators for many years (Junera et al., 1995; Pliss et al., 2005), because the nucleoli are the residences of all expressed rRNA genes (Hadjiolov, 1985) and the latter are assumed to replicate in early S phase (Little et al., 1993; Berger et al., 1997; Li et al., 2005). However, until recently, this assumption was based solely on the established general correlation between early replication timing and the transcriptional activity of gene loci in higher eukaryotes (Goren and Cedar, 2003), which, as is well known, does not apply to all transcribed loci (Taljanidisz et al., 1989; Farkash-Amar and Simon, 2010). Recent work with mouse cells in the Grummt lab produced the first experimental evidence in support of this speculation (Li et al., 2005). The results described here lend further support to this concept through the use of human cells and a different experimental approach. Moreover, the findings of the present report provide a clue to the location of the elusive early-replicating rDNA. I show that replicating rDNA is detectable

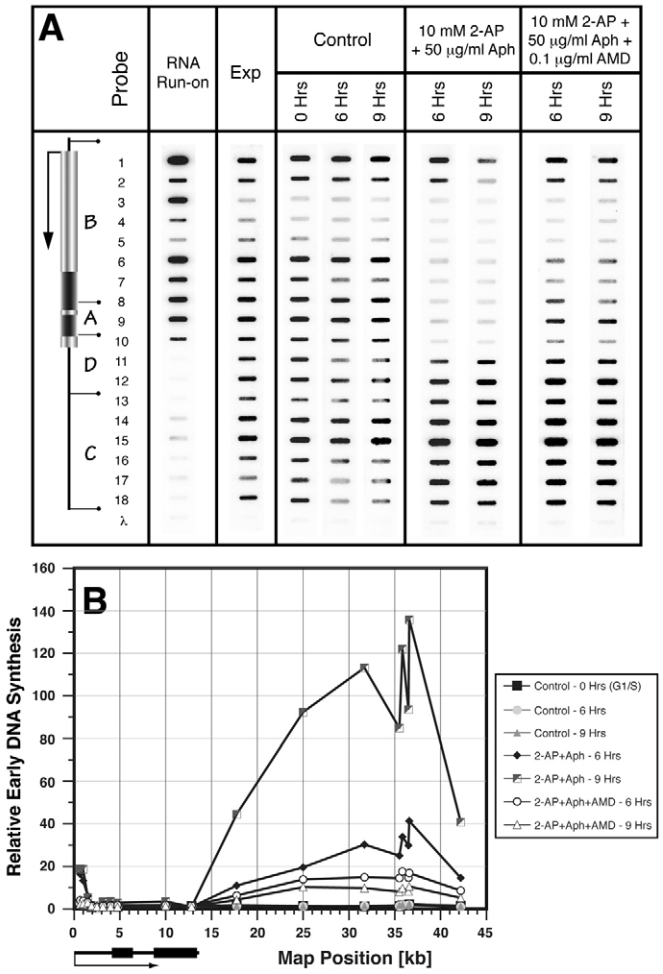

Fig. 6. PolI-mediated transcription sets the replication initiation pattern in the human rDNA repeats during mid and late S phase. (**A**) ^{32}P-labeled nuclear replication run-on products purified from 10^6 HeLa cells synchronized at the G1–S border (0 Hrs) and either released into drug-free medium (Control), or maintained in the presence of Aph + 2-AP alone or in combination with AMD, for 6 or 9 hours were hybridized to a panel of human rDNA probes (1–18; see supplementary material Fig. S7) and a fragment of phage λ DNA (λ). DNA run-on (Exp) and RNA run-on hybridization results obtained with exponentially growing cells are shown as examples of random distribution of replication forks or to highlight the transcribed segment of the rDNA unit, respectively. (**B**) Relative c.p.m. values obtained by phosphoimaging of the blots in A were normalized as in Fig. 5 and plotted against the map position of each probe.

at the periphery of, or outside, nucleoli during the first half of S phase in HeLa cells (Fig. 2A,B; supplementary material Fig. S5A; Table 1) and, following replication, it shifts back to the nucleolar interior and reassociates with UBF (Fig. 4A; supplementary material Fig. S6A), indicating that it mostly represents expressed ribosomal gene copies. Notably, and contrary to the expectations for highly transcribed chromosomal loci (Mesner and Hamlin, 2005), replication initiates randomly throughout the early-replicating rDNA repeats (Fig. 5), which probably represent active rDNA units. The data also show a markedly different spatiotemporal regulation of the silent rRNA genes. The latter replicate during mid and late S phase, mostly in the nucleolar interior in replication factories assembled adjacent to active PolI transcription factories,

with some rDNA replication also detectable at the nucleolar periphery and outside nucleoli at this time. Unexpectedly, replication was found to initiate preferentially in the NTS of the late-replicating rDNA repeats (Fig. 5) and PolI-driven transcription was found to play a role in setting this specific initiation pattern (Fig. 6).

The behavior of the early-replicating rDNA reported here (Figs 2 and 4; supplementary material Fig. S5A and Fig. S6A) might provide a rationale for the unexpected random initiation pattern of the expressed (UBF-associated) rRNA genes. DNA replication in mammalian cells is regulated largely by epigenetic mechanisms, and ongoing transcription is an essential determinant of the preferred replication origin sites (Mesner and Hamlin, 2005; Dimitrova, 2006). When transcription is turned off, replication initiation sites redistribute along the chromatin fiber resulting in random initiation patterns (Dimitrova, 2006). It is therefore possible that replication initiation complexes assembled in the NTSs of active rDNA units during G1 phase redistribute along the nucleosome-free rDNA when it departs from the intranucleolar PolI transcription factories to engage in the perinucleolar replication factories. The organization of active and repressed ribosomal genes in the human NORs has not been elucidated. However, the lack of a good correlation between the number of rDNA copies on individual chromosomes and their transcriptional output (de Capoa et al., 1988) is consistent with individual competent NORs hosting both active and silent rDNA copies. Furthermore, there is some evidence that active human rRNA genes are clustered within competent NORs (Hamkalo and Miller, 1973). Taken together with the lack of preferred initiation sites (Fig. 5), the relatively close origin spacing (Lebofsky and Bensimon, 2005) and the inefficient function of the replication fork barriers (RFBs) reported for human rDNA (Little et al., 1993; Lebofsky and Bensimon, 2005), this organization is compatible with a model in which transcription is turned off in large clusters of active rRNA genes before their spatial relocation and replication. By contrast, unlike the active ribosomal genes, but similar to PolII-transcribed genomic loci (D.S.D. and P. Fraser, unpublished), the silent rDNA copies appear to retain their spatial positioning during transitions between transcription and replication, and they engage in intranucleolar replication factories assembled next to active PolI transcription factories. In yeast NORs, replication always initiates in the NTS downstream of active rRNA genes (Sogo and Thoma, 2004). As shown by the data in Figs 5 and 6, a similar mechanism might operate in human rDNA at least for the silent rRNA genes located in competent NORs. Interestingly, if the different replication initiation profiles uncovered here are conserved in all metazoans, these observations could account for the unexplained lack of correspondence between the low proportion of transcriptionally active rDNA repeats and the higher than expected magnitude of the increase in replication origin specificity following activation of rDNA transcription during early development of *Xenopus* embryos (Hyrien et al., 1995).

Although unexpected, extranucleolar activity involving rDNA is not unprecedented. In yeast, recognition and initial processing of double-strand breaks (DSBs) in rDNA occur inside the nucleolus; however, the lesions are subsequently translocated in the nucleoplasm where they are repaired by the recombination machinery. This transient relocation of DSBs in rDNA is essential to prevent rDNA instability (Torres-Rosell et al., 2007). By a similar logic, it is possible that replicating the active rRNA genes at the periphery, or outside, nucleoli reduces the risk of collisions between the transcription and replication machineries that can cause DSBs and promote rDNA hyper-recombination. The ribosomal genes are the most highly transcribed genomic loci, with 100–150 PolI molecules loaded onto each active gene (Hamkalo and Miller, 1973), and this represents a serious obstacle for their replication. The existence of a robust mechanism, independent of the RFBs, which coordinates the activity of transcription and replication complexes in the competent NORs is implied by the bidirectionality and low efficiency of RFBs in human rDNA (Lebofsky and Bensimon, 2005). Furthermore, replicating the active rDNA copies at the periphery of, or outside, the nucleolar compartment in the absence of transcriptional interference might provide an additional advantage by promoting more efficient rDNA replication, reminiscent of the more synchronous activation of replicons and the higher replication rates of heterochromatin (Comings, 1970). Streamlined replication, on its part, will enable faster switchback to the essential growth-supporting function of the ribosomal genes, namely production of structural rRNAs for building ribosomes (Hadjiolov, 1985).

Materials and Methods

Cell culture and synchronization

HeLa cells were propagated in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) at 37°C in a 5% CO_2 atmosphere. Cell populations synchronized in mitosis or at the G1–S border were prepared as described previously (Dimitrova and Gilbert, 1998; Dimitrova and Gilbert, 1999; Dimitrova and Gilbert, 2000).

For the replication run-on experiments, parallel cultures of HeLa cells, arrested with aphidicolin at the G1–S border, were either released in drug-free medium or maintained in the presence of aphidicolin (Sigma; 50 μg/ml) and 2-aminopurine (Sigma; 10 mM) alone, or in combination with actinomycin D (Sigma; 0.1 μg/ml).

Visualization of replication foci, transcription foci and nuclear proteins

Pulse-labeling of nascent DNA with 5-bromo-2′-deoxyuridine (BrdU; Sigma) and its detection by indirect immunofluorescence were performed as described previously (Dimitrova and Gilbert, 1999; Dimitrova and Gilbert, 2000; Dimitrova, 2006; Dimitrova, 2009). For direct detection of nascent RNA and DNA in situ, HeLa cells grown on coverslips were pulse-labeled for 10 minutes by supplementing the cell culture medium with 100 μM 5-ethynyl uridine (EU; Invitrogen) or 50 μM 5-ethynyl-2′-deoxyuridine (EdU; Invitrogen), respectively. The cells were fixed for 10 minutes with 4% formaldehyde in PBS and permeabilized with 0.5% Triton X-100 in PBS for 10 minutes at room temperature. EU and EdU were stained with Alexa Fluor azide derivatives (Invitrogen) through click chemistry reactions broadly following the manufacturer's recommendations (Dimitrova, 2009). UBF and nucleolin were detected with a rabbit polyclonal antibody (Sigma) or a mouse monoclonal antibody (Santa Cruz Biotechnology), respectively, and appropriate Alexa-Fluor-conjugated secondary antibodies (Invitrogen).

3D DNA FISH

HeLa cells grown on coverslips were fixed for 10 minutes with 4% formaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS for 10 minutes at room temperature and then incubated with RNase A (Sigma; 200–300 μg/ml in PBS) for 2–4 hours at 37°C. DNA FISH was performed as described previously (van Dekken et al., 1988). No FISH signals were detected when digestion with exonuclease III was omitted. For detection of rDNA in situ, *Eco*RI fragments A (7.3 kb) and B (5.8 kb) (supplementary material Fig. S7) (Sylvester et al., 2004) were labeled with digoxigenin-11-2′-deoxyuridine-5′-triphosphate or biotin-11-2′-deoxyuridine-5′-triphosphate by standard nick-translation and detected with sheep anti-digoxigenin antibody (Roche) and 1–2 layers of Alexa-Fluor-conjugated secondary antibodies (Invitrogen), or with Alexa-Fluor-conjugated streptavidin (Invitrogen), respectively.

Laser scanning confocal microscopy

Stacks of serial optical sections (0.25–0.5 μm apart) were collected through the HeLa nuclei sequentially for 3–4 fluorochromes using a Zeiss LSM 510 Meta system housed on an inverted Axiovert 200 microscope equipped with a Plan-Apo 63×1.4 NA oil-immersion objective. The LSM Image Browser (Carl Zeiss) was used to analyze the three-dimensional stacks without further processing. Line-scan plots of fluorescence intensity versus distance were generated from selected optical sections using the RGB Profiler Plugin in ImageJ 1.39. Graphs were created using

DeltaGraph 5 for the Macintosh (Red Rock Software). Figures were assembled from individual images using Adobe Photoshop CS4 and Adobe Illustrator CS4 software.

Nuclear transcription and replication run-on assays

Conditions for nuclear RNA and DNA run-on analysis were as described previously (Dimitrova and Gilbert, 2000; Dimitrova, 2006). Briefly, transcription run-on reactions were performed at 21°C in transcription cocktail supplemented with 25–100 μCi [α-^{32}P]UTP (NEN). For measurements of global transcription rates (2.5×10^5 nuclei per sample), aliquots were removed at various times thereafter and the amount of [^{32}P]UTP incorporated into RNA was determined by acid precipitation. For analysis of rDNA transcription, 1×10^6 nuclei were used per sample and incubated for 30 minutes at 21°C. ^{32}P-labeled RNA was purified and hybridized to a panel of rDNA probes immobilized on nylon membranes (Hybond N+, Amersham-GE Healthcare; 1 μg DNA per slot). All probes (size range 250–2200 bp) were tested by Southern blot analysis for their uniqueness to rDNA and a lack of highly repetitive sequences. A fragment of phage λ DNA was used as a negative control for non-specific hybridization.

In the rDNA replication analysis, for each experimental condition, nuclei were prepared from 1×10^6 cells and incubated for 30 minutes at 21°C in replication cocktail supplemented with 100 μCi [α-^{32}P]-dATP (NEN) to label nascent DNA at existing replication forks. ^{32}P-labeled nuclear replication run-on products were purified and hybridized to the same panel of human rDNA probes.

To correct for differences in probe size, base composition and hybridization efficiency, relative c.p.m. for each probe obtained by phosphoimaging of the blots (Molecular Dynamics) were normalized to the corresponding values for exponentially growing cells as described previously (Dimitrova and Gilbert, 1998; Dimitrova and Gilbert, 2000; Dimitrova, 2006).

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http://jcs.biologists.org/cgi/content/full/124/16/2743/DC1

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