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Replication timing of pseudo-NORs

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ABSTRACT

In mammalian cells, transcriptionally active ribosomal genes are replicated in the early S phase, and the silent ribosomal genes in the late S phase, though mechanisms of this timing remain unknown. UBF (Upstream Binding Factor), a DNA binding protein and component of the pol I transcription machinery, is considered to be responsible for the loose chromatin structure of the active rDNA. Here we question whether such structure alone can ensure early replication of DNA. We investigate this problem on the model of pseudo-NORs, the tandem arrays of heterologous DNA sequence with high affinity for UBF, introduced into human chromosomes. Such arrays are not transcribed, yet efficiently bind UBF and mimic the chromatin structure of active rDNA. In our study, a human derived stable cell line containing one pseudo-NOR on the chromosome 10 was transiently transfected with UBF-GFP and PCNA-RFP, which allowed us to observe in vivo the growth of pseudo-NORs resulted from their replication. We found that replication of pseudo-NORs is not restricted to the early S phase, but continues in the late S phase at a significant level. These results were confirmed in the experiments with incorporation of thymidin analog EdU and BrdU ChIP assay. Similar results were obtained with another cell line containing pseudo-NOR on the chromosome 7. Our data indicate that the specific loose structure of chromatin, produced by the architect protein UBF, is not sufficient for the early replication.

1. Introduction

In eukaryotic cells, replication timing of various genes correlates with their transcriptional activity, chromatin structure, nuclear position, and is regarded presently as an important epigenetic mark (reviewed in Gilbert, 2002; Berezney, 2002; Lucas and Feng, 2003; Hiratani and Gilbert, 2009; Méndez, 2009; Ryba et al., 2010; Pope et al., 2010). Ribosomal genes coding 5.8S, 18S, and 28S rRNA represent special case. These genes exist in numerous copies, some of which are characterized by the 'closed' structure of chromatin and permanent transcriptional silence (Chen and Pikaard, 1997; Raška et al., 2006; Santoro, 2005; Birch and Zomerdijk, 2008; Sanij and Hannan, 2009). It has been found that in mammalian cells transcriptionally active ribosomal genes are replicated predominantly in the early S phase, and the silent ribosomal genes in the late S phase (Berger et al., 1997; Li et al., 2005). However, it remains unclear what determines such timing. One possibility is that the structure of decondensed chromatin characteristic for active ribosomal genes creates favorable conditions for their early replication (Berger et al., 1997; Li et al., 2005).

UBF (Upstream Binding Factor), a DNA binding protein and component of the pol I transcription machinery (Roussel et al., 1996; Hannan et al., 1998; Sirri et al., 2000; Stefanovsky et al., 2001; Russell and Zomerdijk, 2006; Smirnov et al., 2006), is believed to be the major architect of this particular structure (O'Sullivan et al., 2002; Mais et al., 2005; Prieto and McStay, 2007; Wright et al., 2006). We question whether UBF binding alone can determine early replication of DNA, independent of its sequence and transcription status. We investigate this problem on the model of pseudo-NORs (reviewed in Prieto and McStay, 2008). The pseudo-NORs are tandem arrays of heterologous non-transcribed DNA sequence with high affinity for UBF introduced into human chromosomes. Such arrays are not transcribed, yet efficiently recruit UBF to sites outside the nucleolus and, during metaphase, form novel silver positive secondary constrictions. Furthermore, when UBF binds to DNA it recruits pol I and other components of the rDNA transcription machinery, as well as some factors of rDNA processing (Mais et al., 2005; Prieto and McStay, 2007). Thus pseudo-NORs in several aspects mimic the structure

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of the natural clusters of ribosomal genes, or Nucleolus Organizer Regions (NORs).

In the present study, we determined replication timing of pseudo-NORs in two human derived cell lines 3D-1 and 5E-2 with different location of XEn arrays (on the chromosome 10 and 7, respectively) (Mais et al., 2005). We found that replication of these arrays begins in the early, but continues in the late S phase. Our data indicate that the specific loose chromatin structure, produced by UBF binding, does not ensure early replication.

2. Material and methods

2.1. Cell lines and culture

The pseudo-NOR containing cell line 3D-1 is derived from the human fibrosarcoma cell line HT1080 and contains a 1.5-Mb array of XEn sequences, derived from the intergenic spacer of *Xenopus laevis* rDNA, on the long arm of chromosome 10 (Prieto and McStay, 2008). We used also another similar cell line 5E-2 in which the same *XEn* sequences are located on the long arm of chromosome 7 (Mais et al., 2005). The cells were cultivated in Dulbecco's MEM (+Glutamax, sodium pyruvate, and 4.5 g/L glucose; GIBCO) supplemented with 10% fetal bovine serum and 0.1% gentamycin, in atmosphere supplemented with 5% CO_2.

2.2. Plasmids and XEn FISH probe

Plasmids RFP-PCNA and GFP-UBF were kindly provided by Dr. T. Misteli (National Cancer Institute, NIH, Bethesda, MD). The plasmids were transfected into 3D-1 cells using FuGENE (Roche diagnostics).

The plasmid p*XEn8* (Mais et al., 2005) contains a tandem array of eight copies of the 625-bp *X. laevis* enhancer cloned as a SalI–XhoI fragment in a modified pGEM3 vector (Promega).

Applying CLB-Transfection™ Device (Lonza), we introduced the plasmid in *E. coli* cells for amplification. The biotin-labeled XEn probe was prepared using nick-translation kit BIONICK Labeling System (GIBCO-BRL, Invitrogen). The probe was stored in hybridization mixture containing 25 ng of probe, 0.5 mg/ml sonicated salmon sperm DNA, 50% deionized formamide, 2× SSC and 10% dextran sulfate.

2.3. BrdU ChIP

In this assay 3D-1 cells were synchronized by double block with 1 μg/ml aphidicolin (Fluka). Two and five hours after release from the block, the cells were pulse labeled with 30 μM 5′-BrdU (Sigma) for 30 min. Control cells were not labelled. Genomic DNA from the labelled and control cells was isolated using AllPrepDNA/RNAProtein Mini Kit (Qiagen) and sonicated to ~800 bp fragments. 2 μg of heat-denatured DNA from each sample was incubated with 1.5 μg anti-BrdU antibody in PBS (50 μL) for 30 min at room temperature. 10 μg of anti-mouse IgGs in PBS (20 μL) was added and after a further 30 min incubation the mix was centrifuged at 11,000*g* for 10 min. Precipitates were washed twice with 100 μL PBS at room temperature. The final pellets were dissolved by boiling in TE (25 μL). Real Time PCR reactions were performed in duplicate with 2.5 μL of each sample using a DYNAmo HS SYBR Green qPCR mastermix (New England Biolabs) and an MJ research Opticon 2 Thermocycler. The primer pairs employed are as follows:

- rDNA promoter
- Forward Primer: 5′ GTGTGTCCTGGGGTTGACC 3′
- Reverse Primer:5′ GCAGGCGGCTCAAGCAGGAG 3′
- U1 snRNA gene
- Forward Primer: 5′ TTACCTGGCAGGGGAGATAC 3′
- Reverse Primer: 5′ GCAGTCGAGTTTCCCACATT 3′
- Xen sequences
- Forward Primer: 5′ GACCGGGAGTTCCAGGAG 3′
- Reverse Primer:5′ CAGGGCAGGGGGACGAG 3′

2.4. UBF and pol I immunocytochemistry (IC)

Cells were rinsed in PBS and fixed in 2% PFA (formaldehyde freshly prepared from from paraformaldehyde) for 10 min at RT, and permeabilized with 0.2% Triton X-100. Primary antibodies against human UBF and pol I were kindly provided by Dr. U. Scheer (Biocenter of the University of Wurzburg). We also used monoclonal (mouse) anti-UBF antibody (Santa Cruz Biotechnology, Inc.), which binds human UBF. Secondary anti-human and anti-mouse antibodies were labeled with Cy3 or FITC (Jackson ImmunoResearch Laboratories). Coverslips were mounted in Mowiol.

2.5. EdU labeling of replication combined with UBF immunofluorescence

For the labeling of replication, we used the thymidine analogue EdU (5-ethynyl-2′-deoxyuridine) provided by Invitrogen. In contrast to BrdU, detection of EdU requires no DNA denaturation, thus allowing better preservation of the nuclear structure. EdU was administered to the intact living cells in concentration 10 μM for 10 min. The cells were fixed in PFA and processed for UBF IC with FITC-tagged secondary antibody (Jackson ImmunoResearch Laboratories). After rinsing in PBS, the replication signal was visualized using EdU Alexa Fluor® 647 Imaging Kit (Invitrogen). We also labeled replication sites with 20 μM BrdU (Sigma, Aldrich). The signal was visualized by using mouse anti-BrdU antibody (Roche).

2.6. Combined UBF immunofluorescence and XEn FISH

After UBF immunolabeling the cells were postfixed with methanol/acetic acid (3:1) overnight at −20 °C, then the regular FISH followed (Pliss et al., 2005). The UBF signal was well preserved due to this procedure. For denaturation, cells were placed in 70% formamide/2× SSC for 3 min at 73 °C followed by 1 min in 50% formamide/2× SSC at 73 °C. Hybridization proceeded in wet chamber with 50% formamide at 37° overnight. Post hybridization washing was performed after Harničarová et al. (2006). Namely, the cells were washed in 50% formamide in 2× SSC, pH 7, for 15 min at 43 °C, in 0.1% Tween-20/2× SSC for 8 min at 43 °C; in 0.1% Igepal (ICN Biomedicals, Inc)/4× SSC for 3 × 4 min at 37 °C, in PBS 3 × 3 min at RT. XEn FISH probe was visualized using rabbit anti-biotin antibody (Bethyl).

2.7. Image analysis

For assessing colocalization between UBF and EdU signals, confocal image stacks were recorded under conditions of optimal sampling (Heintzmann, 2006), then deconvolved using Huygens professional software (Scientific Volume Imaging) with a calculated point spread function (PSF) and the classical constrained maximum likelihood estimation (CMLE) algorithm. We also examined our samples using a newly constructed widefield microscopy system consisting of an inverted microscope (IX71 with a 100×, 1.35NA PlanApo objective, Olympus, Hamburg, Germany), EMCCD camera (Ixon DU885, Andor), Piezo Z stage (Nanoscan Z, Prior Scientific), and appropriate filters sets for FITC and Cy5. The acquisition system is controlled by IQ software (Andor). The microscope is supported on a large, actively isolated optical table (Thor Labs)