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Upstream binding factor-dependent and pre-rRNA transcription-independent association of pre-rRNA processing factors with rRNA gene

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ABSTRACT

The nucleolus is the ribosome biogenesis center. The nucleolar structure is disrupted upon entry into mitosis and is formed in early G1 phase. To understand the molecular mechanisms of nucleolar assembly and disassembly, we have studied the mechanism of association between factors involved in pre-ribosome RNA (rRNA) processing and rRNA gene chromatin (r-chromatin). We found that the pre-rRNA transcription-processing linking factor Nopp140 and pre-rRNA processing factors such as DKC1 and fibrillarin (FBL) associate with r-chromatin during interphase, while Nopp140, DKC1, and FBL were released from r-chromatin in mitosis. The association of these factors with r-chromatin was found to be restored independent of pre-rRNA transcription in early G1 phase, but a mature nucleolar structure was not formed, suggesting that nucleolar assembly can be divided into at least two steps with respect to pre-rRNA transcription. Moreover, we found that the r-chromatin association of Nopp140, DKC1, and FBL was dependent on the transcription factor upstream binding factor (UBF). However, we demonstrated that UBF alone was not sufficient to recruit these pre-rRNA processing factors to r-chromatin. Thus, UBF is necessary but not sufficient for the associations between pre-rRNA processing factors and r-chromatin.

1. Introduction

The primary function of the nucleolus is ribosome biogenesis, which includes ribosomal RNA precursor (pre-rRNA) transcription, modifications, and processing [1]. The 47S pre-rRNA is synthesized by RNA polymerase I (pol I). The transcribed 47S pre-rRNA is modified and processed successively by a variety of RNA–protein complexes (ribonucleoprotein, RNP).Modifications of pre-rRNA consisting of methylation and pseudouridylation are directed by two classes of small nucleolar RNAs (snoRNAs), box C/D and box H/ACA snoRNAs, respectively. Box C/D snoRNAs associate with four proteins, 15.5K, NOP56, NOP58, and fibrillarin (FBL), while box H/ACA snoRNAs associate with GAR1, NHP2, NOP10, and DKC1 (also known as dyskerin/NAP57) [2]. DKC1 was originally identified as a Nopp140-associated protein [3]. Nopp140 is a phosphoprotein localized in the nucleolus and Cajal body; it interacts with both box C/D snoRNPs and box H/ACA snoRNPs and may function as a snoRNP chaperone [4,5]. In addition, because Nopp140 was shown to associate with RNA polymerase I subunits [6], Nopp140 is suggested to link pre-rRNA transcription and processing.

The nucleolar structure is disrupted upon entry into mitosis and is formed in early G1 phase. During mitosis, pre-rRNA transcription and processing are silenced. Before nucleolar assembly, pre-rRNAs and factors involved in pre-rRNA modifications and processing form RNPs and are accumulated in small granules (prenucleolar body, PNB) outside the nucleolus. These RNPs are then recruited to chromosome regions termed nucleolar organizer regions (NORs) containing rRNA gene repeats to form the mature nucleolar structure [7]. However, the molecular mechanism by which these RNPs are assembled to NORs remains unknown. A previous study indicated that mature nucleolar structure formation is impaired but pre-rRNA processing factors are accumulated in segregated nucleoli when mitotic cells are released into G1 phase in the presence of a Pol I inhibitor [8,9]. However, it is currently unknown the assembly processes of pre-rRNA processing factors to NORs. It was also

Abbreviations: rRNA, ribosomal RNA; pre-rRNA, ribosomal RNA precursor; Pol I, RNA polymerase I; NOR, nucleolar organizer region; PNB, prenucleolar body; r-chromatin, rRNA gene chromatin; RNP, ribonucleoprotein; snoRNA, small nucleolar RNA; Act D, actinomycin D.

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reported that the transcription factor upstream binding factor (UBF) has the ability to recruit pre-rRNA transcription factors and factors linking pre-rRNA transcription and processing [10]. Thus, it is likely that UBF plays a crucial role in the formation of the nucleolus. Although immunofluorescence-based analyses have provided a clue to understand the nucleolar assembly and disassembly mechanism, physical association between r-chromatin and pre-rRNA processing factors have not been well understood. Here, we studied the association between pre-rRNA processing factors and r-chromatin to understand the molecular mechanism of nucleolar assembly and disassembly. We revealed that pre-rRNA processing factors are associated with r-chromatin in interphase and are released from r-chromatin in mitosis. We also found that the association of pre-rRNA processing factors with r-chromatin was restored in the presence of a pre-rRNA transcription inhibitor in early G1 phase. Furthermore, UBF was found to play an important role in the association of pre-rRNA processing factors with r-chromatin. Our results contribute to the understanding of the regulatory mechanism of nucleolar assembly and disassembly.

2. Materials and methods

2.1. Cell culture, transfection, cell cycle synchronization, and 5-fluorouracil incorporation assay

HeLa and U2OS cells were maintained in Dulbecco modified Eagle medium (Nacalai Tesque) containing 10% (v/v) heat-inactivated fetal bovine serum. U2OS 2–6–3 cells were maintained in the same medium supplemented with 50 μg/mL hygromycin B (Invivogen). Transient transfection of plasmid DNA was performed using GeneJuice (Novagen) according to the manufacturer's instructions. Small interfering RNA (siRNA) was transfected using Lipofectamine RNAi MAX (Life Technologies). Stealth RNAs for negative controls (Stealth RNAi negative-control duplex, catalog No. 12935-300, Life Technologies), UBF and RRN3siRNAs (UBTF-HSS111143 and RRN3-HSS123162 from Life Technologies) were used. To prepare mitotic cells, cells were incubated in medium containing 2 mM thymidine (Sigma) for 24 h, released into medium without thymidine for 3 h, and then incubated for additional 12 h in the presence of 0.5 μg/ml of nocodazole. Mitotic cells were collected by shaking dishes. For the experiment shown in Figs. 2 and 3, mitotic cells were released into culture medium in the absence or presence of 50 ng/mL of actinomycin D (Sigma). In 5-fluorouracil (5-FU) incorporation assay, cells were incubated for 60 min in the presence of 3 mM 5-FU (Sigma).

2.2. Antibodies

The following antibodies were used in this study: mouse monoclonal antibodies for β-actin (C4, Santa Cruz Biotechnology), BrdU (BU-33, Sigma), and RPA194 (C-1, Santa Cruz Biotechnology), rabbit polyclonal antibodies for dyskerin (DKC1) (H-300, Santa Cruz Biotechnology), fibrillarin (FBL) (H-140, Santa Cruz Biotechnology), histone H3 (ab1791, Abcam), Nopp140 (H-80, Santa Cruz Biotechnology), phospho-histone H3 (Ser10) (Millipore), RRN3 (ab112052, Abcam; Y-23, Santa Cruz Biotechnology), and UBF (H-300, Santa Cruz Biotechnology).

2.3. Chromatin immunoprecipitation

Chromatin extracts were prepared from cells fixed with 1% (v/v) formaldehyde by extensive sonication. In all ChIP assays, DNA

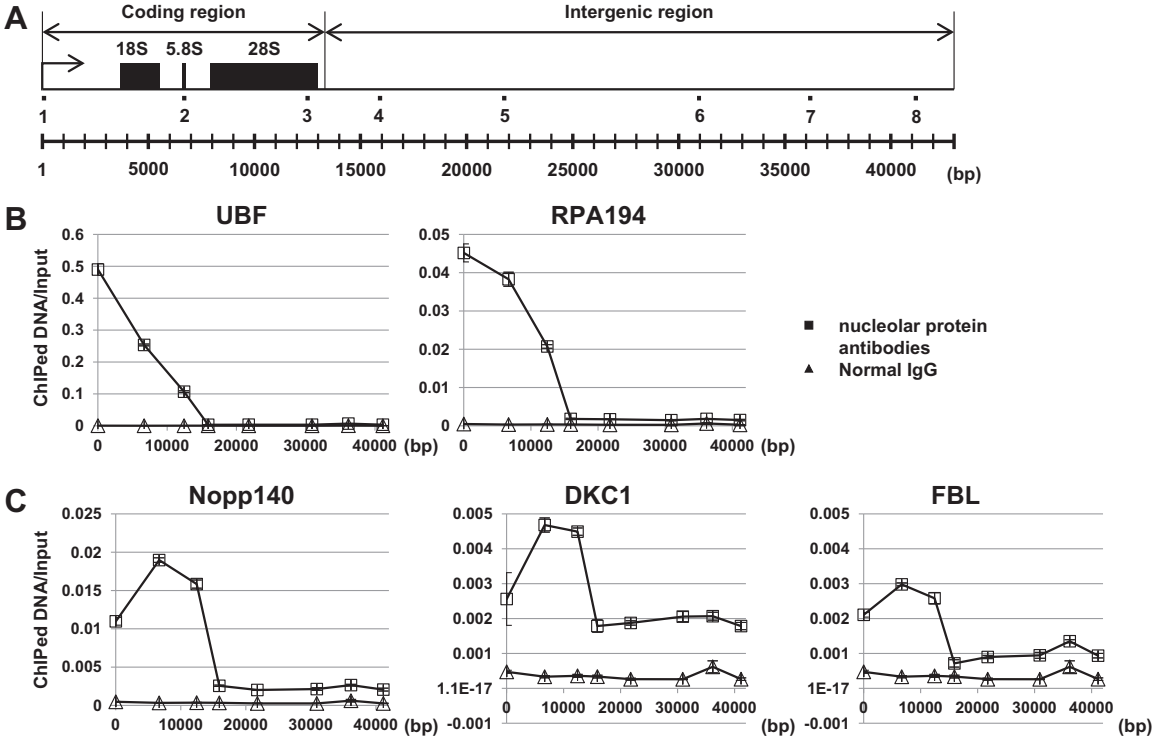

Fig. 1. Pre-rRNA processing factors were associated with r-chromatin in asynchronous cells. (A) Schematic representation of the human rRNA gene. The positions of 18S, 5.8S, and 28S rRNAs are shown schematically by black bars. Primer positions used for ChIP assay are shown by 1–8. Pre-rRNA transcription start site is set as 1. Primer sequences are listed in Table S1. (B and C) The r-chromatin association of nucleolar proteins in asynchronous. ChIP assays using antibodies against UBF and RPA194 (B), Nopp140, DKC1, and FBL (C) were performed with extracts derived from HeLa cells. Normal IgGs were used as controls. The amounts of precipitated DNAs relative to those of input DNAs were quantitatively analyzed with specific primer sets (1–8) for the rRNA gene shown in (A). Q-PCRs were performed in triplicate and error bars represent S.D. in one experiment. Two independent experiments showed similar results.