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Research paper

Cell cycle and growth stimuli regulate different steps of RNA polymerase I transcription

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ABSTRACT

Transcription of the ribosomal RNA genes (rDNA) by RNA polymerase I (Pol I) is a major control step for ribosome synthesis and is tightly linked to cellular growth. However, the question of whether this process is modulated primarily at the level of transcription initiation or elongation is controversial. Studies in markedly different cell types have identified either initiation or elongation as the major control point. In this study, we have re-examined this question in NIH3T3 fibroblasts using a combination of metabolic labeling of the 47S rRNA, chromatin immunoprecipitation analysis of Pol I and overexpression of the transcription initiation factor Rrn3. Acute manipulation of growth factor levels altered rRNA synthesis rates over 8-fold without changing Pol I loading onto the rDNA. In fact, robust changes in Pol I loading were only observed under conditions where inhibition of rDNA transcription was associated with chronic serum starvation or cell cycle arrest. Overexpression of the transcription initiation factor Rrn3 increased loading of Pol I on the rDNA but failed to enhance rRNA synthesis in either serum starved, serum treated or G0/G1 arrested cells. Together these data suggest that transcription elongation is rate limiting for rRNA synthesis. We propose that transcription initiation is required for rDNA transcription in response to cell cycle cues, whereas elongation controls the dynamic range of rRNA synthesis output in response to acute growth factor modulation.

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1. Introduction

Transcription of the ribosomal RNA (rRNA) genes that encode the precursor of the 28S, 5.8S and 18S rRNAs is generally considered one of the most fundamental rate-limiting steps for the synthesis of ribosomes and therefore growth and proliferation (Hannan et al., 1998a; Moss and Stefanovsky, 2002; Kusnadi et al., 2015; Ruggero and Pandolfi, 2003; White, 2005). Human diploid cells have 400–600 rDNA repeats (Zentner et al., 2011; Stults et al., 2008) but only a subset of these genes is transcribed at any given time (Hamperl et al., 2013; Poortinga et al., 2014; Sanij and Hannan, 2009; Sanij et al., 2008; Conconi et al., 1989). Modulation of rRNA synthesis rates in response to growth factors, stress, and cell cycle cues appears to be controlled almost exclusively by changing the activity of a fixed number of "transcriptionally competent genes" rather than epigenetically silencing or reactivating rRNA genes (Grummt and Pikaard, 2003; Stefanovsky and

Abbreviation: +S, With serum; −S, Without serum; CHX, Cycloheximide; DMEM, Dulbecco's modified Eagle's medium; ECL, Enhanced chemiluminescence; ETS, Externally transcribed spacer; Exp, Exponentially growing cells; FBS, Fetal bovine serum; h, Hour; LY, LY294002; MFP, Mifepristone; PI3K, Phosphatidylinositol-3-Kinase; PIC, Preinitiation complex; Pol I, RNA polymerase I; qChIP, Quantitative chromatin immunoprecipitation; qRT-PCR, Quantitative real-time PCR; rapa, Rapamycin; rDNA, Ribosomal RNA gene; rRNA, Ribosomal RNA; SD, Standard deviation; s.e.m., Standard error of the mean; SL-I, Selectivity factor 1; UBF, Upstream binding factor.

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Moss, 2006). Pol I transcription initiation begins with the formation of the preinitiation complex (PIC) by the upstream binding factor (UBF) and the TBP-containing complex selectively factor (SL-1) at the rDNA promoter (Comai et al., 1992; Comai et al., 1994; Gorski et al., 2007; Heix et al., 1997; Zomerdijk et al., 1994). The resultant UBF/SL-1 complex facilitates recruitment of an initiation competent subpopulation of Pol I, defined by the presence of the basal regulatory factors PAF53/PAF49 and Rrn3 (also called TIF-1A), to form a functional PIC at the rDNA promoters (Moss and Stefanovsky, 2002; Beckmann et al., 1995; Friedrich et al., 2005; Goodfellow and Zomerdijk, 2013; Hempel et al., 1996; Schnapp et al., 1994; Stepanchick et al., 2013).

Rrn3 appears to play an essential role in integrating extracellular cues with transcription initiation. Phosphorylation of Rrn3 by kinases linked to nutrient or energy availability, mitogen activation, stress and cell cycle cues modulate its transcriptional initiation activity. These observations led to the proposal that transcription initiation *via* modulation of Rrn3 activity is the major rate-limiting step for rRNA synthesis in mammalian cells (Goodfellow and Zomerdijk, 2013; Hoppe et al., 2009; Mayer et al., 2005; Mayer et al., 2004; Zhao et al., 2003; Bierhoff et al., 2008; Hannan et al., 2011). This model is supported by a study that utilized live cell imaging combined with computational modeling to analyze the transcription complex dynamics of rDNA transcription regulation during the cell cycle. Using this approach it was demonstrated that activation of rDNA transcription *in vivo* occurs *via* modulation of the efficiency of PIC assembly *i.e.*, through changes in transcription initiation (Gorski et al., 2008).

Emerging evidence however, suggests that the elongation step in Pol I transcription is important for the overall control of rRNA synthesis rate as well as ensuring efficient rRNA processing (Kopp et al., 2007; Schneider, 2012; Schneider et al., 2006; Schneider et al., 2007; Stefanovsky et al., 2006). In contrast to the model of initiation regulation, Stefanovsky et al., demonstrated that transcription elongation, regulated through cyclic phosphorylation of UBF, was the dominant event in growth factor regulation of the rRNA genes (Stefanovsky et al., 2006). However, the question of whether transcription is modulated primarily at the level of initiation or elongation remains unclear.

As the various studies on the limiting steps for rRNA synthesis have been carried out with diverse cell types and regulatory conditions it is difficult to directly compare the data. In this study, using NIH3T3 fibroblasts as a model system, we have indirectly determined initiation and elongation rates using a combination of metabolic labeling of the 47/45S rRNA, quantitative chromatin immunoprecipitation (qChIP) analysis to measure loading of Pol I on the transcribed region of the 47S rRNA genes and overexpression of the transcription initiation factor Rrn3 to identify possible rate-limiting steps for rRNA synthesis under different physiological states.

Our data demonstrate that rDNA transcription rate can be modulated over a wide range in response to acute manipulation of serum levels without changing Pol I loading. Thus, serum stimulation modulates Pol I elongation. In contrast, chronic serum removal and arrest in G0/G1 was associated with decreased Pol I loading, thus impaired initiation. Overexpression of the initiation factor Rrn3 under serum starvation conditions led to restored Pol I loading across the 47S rRNA gene to levels similar to those observed in exponentially growing cells. This is consistent with the model that increased expression of Rrn3 was sufficient to overcome the block in transcription initiation observed during extended serum removal. However, Rrn3 overexpression did not induce rRNA synthesis in either serum starved, serum stimulated nor in G0/G1-arrested cells due to an apparent block in elongation. Thus our data suggest Pol I elongation to be limiting for efficient rDNA transcription in both acutely and chronically serum-deprived cells.

These data therefore support a model whereby Pol I elongation controls the dynamic range of rRNA synthesis output in response to serum modulation.

2. Materials and methods

2.1. Cell culture and cell lines

NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) at 37 °C. Inducible wild type Rrn3 was expressed in NIH3T3 cells using GeneSwitch system (Invitrogen). Cells were maintained as above in the presence of 50 μg/mL hygromycin (Invitrogen) and 100 μg/mL zeocin (Invitrogen). Rrn3 expression was induced by the addition of mifepristone (MFP, Sigma). Cells were made quiescent by serum deprivation in 0.5% bovine serum albumin (Sigma) supplemented DMEM and re-fed with DMEM containing 10% FBS.

2.2. Psoralen crosslinking

Psoralen crosslinking was performed as described in (Sanij et al., 2008; Conconi et al., 1989). Briefly, 5×10^6 cells were scraped in 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40, nuclei were pelleted (1500 rpm, 4 °C 10 min) and resuspended in (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA), and irradiated in the presence of 4,5,8′-trimethylpsoralen (Sigma) with a 366-nm UV light box at a distance of 6 cm. 200 μg/mL psoralen was added at a 1:20 dilution every 4 min for a total irradiation time of 20 min. Genomic DNA was isolated, digested with *Sal* I, and separated on a 0.9% agarose gel, and alkaline Southern blotting was performed. To reverse psoralen cross-linking, filters were treated with 254-nm UV rays at 1875×100 μJ/cm^2 using a UV cross-linker (Stratalinker 2400; Agilent Technologies). The membrane was then hybridized to a purified ^{32}P (Amersham)-labeled nick-translated rDNA fragment (−167 to +293 nucleotides relative to transcription start site), hybrids visualized by scanning on a PhosphoImager (GE Healthcare), and quantitated using ImageQuant (TLv2005.04; GE Healthcare).

2.3. RNA extraction and cDNA synthesis

Cells were lysed in 4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M β-mercaptoethanol and RNA extracted according to standard methods. First-strand cDNA was synthesized using random hexamer primers and reverse transcriptase (Invitrogen) as per manufacturer's protocol. Primer sequences are listed in Supplementary Table 1.

2.4. Ribonuclease protection assay

Ribonuclease protection assays were performed as described in (Lister et al., 2006). 400 to 600 pg of ^{32}P-riboprobes against the +55 to +155 region of the 5`ETS of mouse rDNA and RNA samples from equivalent cell numbers were made up in 80% (v/v) deionized formamide, 40 mM PIPES pH 6.7, 0.4 M NaCl, 1 mM EDTA, denatured at 85 °C and hybridized at 45 °C overnight. Un-hybridized RNA was digested with 10 μg RNase A (Roche) and 50 U RNase T1 (Roche). After denaturation, hybridized RNA was separated on a 5% non-denaturing polyacrylamide gel. The gel was dried and visualized by scanning on a PhosphorImager (Fuji imaging plate, Type Bas-IIIs, Fuji Photo Film). The images were then quantified with ImageQuant (TLv2005.04; GE Healthcare).

2.5. Metabolic labeling to measure rRNA synthesis

As described in (Chan et al., 2011), cells were cultured in phosphate-free media (Gibco) 1.5 h prior to the addition of 500 μCi ^{32}P-orthophosphate (MP Biomedicals) for 30 min and chased for 1 h with non-radioactive media. Total RNA was extracted and 5– 10 μg of RNA was separated on a 1.2% MOPS-(7.2%) formaldehyde agarose gel. The gel was dried and visualized by scanning on a phosphor-imaging screen

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