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"Ribosome Biogenesis: Streamlining the Genome for the Efficient Production of this Biological Nanomolecular Machine"

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ABSTRACT

One of the most complex nanomolecular machines found within the cell is the ribosome. Integral to translation, the ribosome is conserved on a functional level across all domains of life. The eukaryotic ribosome is comprised of approximately 80 Ribosomal Proteins (RPs) and four rRNAs that are highly processed, folded, and assembled by more than 200 processing and assembly factors (termed rRNA and ribosome biogenesis factors). The cell requires roughly stoichiometric levels of each of these components to meet cellular demand for protein synthesis, to maintain fidelity of this process, and to ensure that faithful translation occurs. Ribosome biogenesis is an energetically consumptive process, and there are many mechanisms the cell employs in order to properly balance expression of the requisite components. The failure to properly regulate this process results in cellular dysfunction, in higher eukaryotes it can lead to disease such as various cancers. This commentary will discuss recent developments in the understanding of the role that spatial positioning – the linear arrangement of genes along the chromosome throughout the genome – plays in the regulation of ribosome biogenesis, focusing on lessons learned from the budding yeast, *Saccharomyces cerevisiae*, and their implications in higher eukaryotic organisms.

Introduction

Fundamental to our understanding of molecular biology is the 'Central Dogma', describinghow the flow of sequential information stored in DNA is transcribed into an RNA intermediary and subsequently translated into a corresponding protein (or proteins) [1]. While this model has increased in complexity, it still represents the foundation for our current understanding of molecular biology. Central to the process of translation is the ribosome, the ribonucleoprotein macromolecular complex which functions to 'read' a mRNA and catalyze the synthesis of a polypeptide from amino acid monomers. As a nanomolecular machine, the ribosome is conserved across all domains of life on a functional level, catalyzing the peptidyl-transferase reaction in all species, from the simplest of prokaryotes to the most complex eukaryotic organisms. Although variations exist in the composition of the ribosomes found between different organisms, there are ubiquitous components and similarities. Thus, observations from many model organisms shed insight into the many complexities of this nanomolecular machine [2, 3]. Study of the ribosome in the

budding yeast, *Saccharomyces cerevisiae*, serves as a model for understanding the complexities ribosome biogenesis and its regulation in eukaryotes [4].

An Overview of Ribosome Biogenesis inBudding Yeast

The budding yeast ribosome is comprised of four rRNAs, which themselves are composed of roughly 5,500 ribonucleotides and 80 ribosomal proteins (RPs), encoded throughout the genome by the 137RP genes. The production, assembly, and maturation of each ribosome requires the activity of rRNA and ribosome biogenesis (RRB) factors andalso of 75 small, nucleolar RNAs (snoRNAs)[5]. Ribosome biogenesis requires coordinating the action ofall three RNA polymerases: RNA polymerase I transcribes the 35S pre-rRNA, RNA polymerase II transcribes the RP, RRB, and a subset of the snoRNA genes, and RNA polymerase III transcribes the 5S pre-rRNA and a subset of the snoRNA genes [4].

Assembly of the ribosome requires extensive pre-rRNA modification by a multitude of factors. This includes processing by small, nucleolar ribonucleoproteins (snoRNPs, named for their corresponding snoRNA components), antisense RNAs acting as enzymatic guides, which are responsible for pseudouridylation at 44 nucleotides(by the box H/ACA snoRNPs), 2'-O-ribose methylation at 67 nucleotides (by the box C/D snoRNPs). In yeast, these work with nine methyltransferases and an acetyltransferase to modify roughly 2% of the rRNA nucleotides [6]. There is rRNA cleavage by endonucleases and exonucleases toremove the internal and external transcribed spacers and many helicases, nucleotide binding proteins, GTPases, and ATPasesworking together in concert [4, 5].

Concurrent with this processing is the folding of rRNAs and the incorporation of the RPs as the pre-ribosomal particles mature into functional ribosomes. All of the proteins that modify the rRNAs and help to fold and assemble them with the RPs are members of the RRB family. The RRB proteins are components of: the RNA polymerase I and III holoenzyme complexes, the snoRNPs, the nucleases, the helicases, and all of the assembly factors –comprising a gene family of over 200 members [4, 7, 8].

As a whole, ribosome biogenesis proves an energetically-consumptive process. There are roughly 200,000 ribosomes per yeast cell. During periods of rapid growth (logarithmic phase growth) this corresponds to the production of mature, functional ribosomes at a rate of roughly 2,000 ribosomes per minute to meet cellular demand for protein synthesis [9]. This result in tight regulation of each of these components, especially during times of nutrient deprivation or during adaptation to stressors – the production of ribosomes is rapidly downregulated to allow the cell to allocate the cellular resources that are available to survival and establishment of homeostasis [10, 11].

The Regulation of Ribosome Biogenesis in Budding Yeast

S. cerevisiae balances growth rate with available nutrients, such as carbon and nitrogen, via the well-conserved 'Target of Rapamycin' (TOR) and 'Protein Kinase A' (PKA) intracellular signaling pathways [12-14].As long as nutrients are plentiful these pathways remain active, turning on ribosome biogenesis via signal transduction through the activity of intracellular kinases such as Sch9 and Sfp1[5]. The major level of regulation of ribosome biogenesis occurs at the level of transcription, and these signaling pathways ultimately converge to activate transcription of all the necessary components simultaneously.

The ribosomal DNA (rDNA) comprises roughly 10% of the budding yeast genome and isfound asa tandem array of between 100-200 repeats clustered on chromosome XII, where itis sequestered into the nucleolus [9]. Transcriptional regulation of the rDNA repeats occurs by: TOR complex 1 (TORC1), Hmo1, inhibition of Maf1, inhibition of the Rpd3 lysine deacetylase complex (KDAC), and chromatin remodeling complexes – such as the Tof2-Lsr4/Csm1 and RENT complexes [4, 10, 15]. The level of expression is modulated by altering the ratio of actively transcribedversus repressed repeats, matching transcription to cellular growth rate. Thus the

rDNA repeats alternate between an active and inactive transcriptional state to accommodate cellular demand [4]. Thisarrangement of the rDNA clustered as tandem repeatsalong the chromosome and within the nucleolus allows for efficient transcription through the localization of regulatory proteins.

(Stb3) and Tod6 and Dot6, respectively [8,19-21].The snoRNA genes are enriched for promoter motifs for Tbf1, Reb1, and the TATA box and many of these can be found within introns of ribosomal proteins and other genes involved in translation [14].

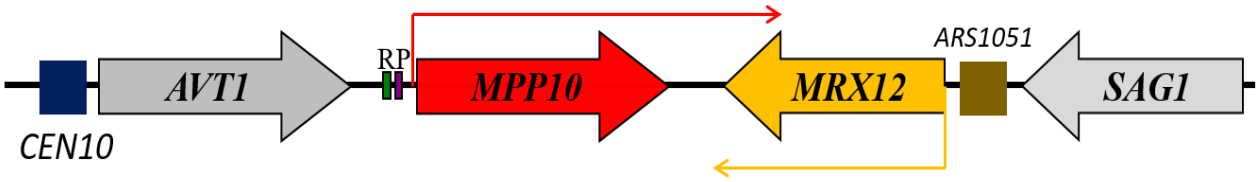

Figure 1: The rRNA and ribosome biogenesis gene pair *MPP10-MRX12* and surrounding locus.
The RRB gene pair found in the pericentromeric region on Chromosome X. Arrow orientation represents the direction of transcription of each gene, and R=RRPE and P= PAC promoter motifs.

Both the RP and the RRB genes are distinct regulons.A relatively underappreciated differencebetweeneach familyis that the cell requires roughly stoichiometric levels of both regulons – but different absolute levels of expression are needed between each set of these gene families. The RPs is transcribed, translated, and incorporated into the ribosome as it matures, remaining an integral component of the ribosome throughout its life. On the other hand the RRBs do not remain with the ribosome after maturation [4]. Many of the RRB genes code for proteins acting as enzymes whereby theyperform their functions repeatedly and thus the cell needs lower transcriptional levels of the RRB gene family. Therefore the RP genes are expressed at significantly higher levels than the RRB genes, which occurs viaa combination of distinct promoter architectures and DNA binding proteins for each gene family [14].

The RP gene family is characterized by promoters containing binding sites for the Repressor Activator Protein 1 (Rap1), Forkhead-Like protein (Fhl1), Ifhl, Abf1, Hmo1 and nucleosome excluding dA:dT tracts[14, 17]. Whereas the RRB gene family is enriched for the ribosomal RNA Processing Element (RRPE) and the polymerase A and C (PAC) promoter motifs [8,18]. These subsequently bind to the sin-three binding protein

Functional Clustering Allows For Adjacent Gene Co-Regulationin Budding Yeast

Despite the differences inherent among these gene families, a non-random genomic distribution of both the RP and the RRB family members exists throughout the genome [8,22]. The RP genes and the RRB genes are found clustered together, primarily in pairs throughout the genome, andthis spatial arrangement gives rise to 'adjacent gene co-regulation' [22]. The canonical example for these transcriptional phenomena is the RRB gene pair, *MPP10-MRX12* (Figure 1). These genes are both co-regulated members of the RRB regulon and are located on Chromosome X in a convergent orientation within the pericentromeric region. Bioinformatic analysis of this locus identified the PAC and the RRPE motifs (the two promoter motifs that are characteristic of the RRB regulon) [8]. Surprisingly, both motifs were identified directly upstream of *MPP10* alone – the area upstream of *MRX12* is an active Autonomously Replicating Sequence (ARS) and lacked these motifs.

Taking advantage of the malleable genetic background of *S. cerevisiae*, functional dissection of the relationship of this gene pair was performed. A series of promoter mutantswere generated whereby the RRPE, the PAC, or both motifs upstream of *MPP10* were scrambled, and it

was observed the coordinated expression of both *MPP10* and *MRX12* was uncoupled from the other members of the RRB regulon during the stress response and upon glucose replenishment [22]. Likewise, this resulted in a failure to recruit the RRPE binding factor, Stb3, to the promoter of *MPP10* and caused a loss of changes in histone acetylation in the promoter of *MPP10*.The promoter area directly upstream of *MRX12*remainedunaffectedwith regards to histone acetylation and Stb3 recruitment regardless of the genetic background [22]. This surprising result demonstrated a significant regulatory role for the two promoter motifs upstream of *MPP10*, including a role in the transcription of *MRX12*from approximately four kilo bases away (a significant genomic distance in *S. cerevisiae*).

Several follow-up studies focused on the need for adjacency in the regulation of the*MPP10-MRX12* gene pair.Separation of this gene pair via the integration of areporter construct, *KAN^R-klURA3*, into the intergenic region at this locuscompletely uncoupled the expression of *MRX12* from that of *MPP10* – the latter of which remained co-regulated with the rest of the RRB gene family [22]. A subsequent study focused on the physical separation of *MPP10-MRX12* with an inducible *LEU2* construct, which allowed for activation or repression of the construct by simply altering the growth conditions.When the *LEU2* gene was actively transcribed, the expression of *MRX12* was uncoupled from that of *MPP10* and the rest of the RRB regulon. However, when *LEU2* was repressed, *MRX12* remained co-regulated with *MPP10* – in spite of the additional distance separating this gene pair [23].

Functional Clustering of RPs and RRBs Streamlines the Genome to CoordinateTranscription and is a Characteristic of Eukaryotic Genomes

Subsequent research utilized a bioinformatics approach to compare the transcriptional similarity of the functional clusters to the members of the same gene family that are not found in clusters (termed singletons). In both the RP and RRB regulons the functional clusters exhibit a higher Pearson's correlation coefficient (PCC) (i.e. greater transcriptional similarity) compared to the unpaired singletons within each family during the stress-response induced by heat shock, osmotic shock, and by the induction of DNA damage. Additionally, functionally-clustered genes exhibit a higher PCC during the expressional changes occurring throughout the cell cycle [24]. This suggests the functionally clustered genes adjacent to each other allow for a more efficient transcriptional regulation than the singletons.

This finding that functional clustering of both ribosomal proteins and rRNA and ribosome biosynthesis factors is prevalent in *S. cerevisiae* leads to the question of the wide spread nature of this phenomena. Initial comparative analysis focused on conservation of functional clustering of both the RP and RRB genes within divergent fungal lineages. Investigation of the genomic distribution of both gene families in both *Candida albicans* and *Schizosaccharomyces pombe* found that both species exhibit a non-random genomic distribution of both gene families (Arnone 2011).In *C. albicans,* 46 out of the 168 RRB genes and 25 out of the 118 RP genes are found in functional clusters (p-value for this distribution = $2.3x10^{-15}$ and p-value = $1.4x10^{-14}$, respectively). In *S. pombe* 20 out of the 97 RRB genes and 26 out of the 166 RP genes are clustered (p-value = $1.2x10^{-10}$ and p-value = $1.2x10^{-5}$, respectively) [21]. This arrangement was then characterized through the use of a comparative genomics across a broader range of fungi. While a significant fraction of both gene families found clustered in each of the fungal lineage explored, the actual paired genes found in clusters were not the same [24]. Using the *S. cerevisiae* clusters as a reference point, the number of identical clusters found decreases with evolutionary distance. In *S. pombe* there are no conserved RP-RP or RRB-RRB functional clusters from *S. cerevisiae*, even though the absolute levels of clustered genes remains a constant within this species [24]. This suggests a genomic distribution that has arisen multiple times in different species, rather than representing an ancestral relationship maintained over time.

Significant levels of functional clustering for the RPs and the RRB gene families are found in many higher

eukaryotic organisms. Expansion of the genomic comparisons to more divergent fungi, single-celled organisms, and more complex multicellular eukaryotes conclusively found that functional clustering is a conserved genomic organizational strategy.Statistically significant, non-random genomic distribution of RP genes organized into functional clusters were seen in: *Neurospora crassa* (p-value: $3.9x10^{-15}$), *Aspergillus nidulans* (p-value:$1.5x10^{-7}$), *Arabidopsis thaliana* (p-value: $1.0x10^{-14}$), *Caenorhabditis elegans* (p-value:$1.1x10^{-5}$), *Tetrahymenathermophile* (p-value: $1.9x10^{-8}$), *Plasmodium falciparum* (p-value: $2.4 x10^{-4}$), and *Homo sapiens* (p-value: $2.8x10^{-3}$).A non-random distribution of RRB genes were observed in: C. *elegans* (p-value: $1.6x10^{-4}$), *A. thalania* (p-value: $6.9 x10^{-2}$), *T. thermophile* (p-value: $2.4x10^{-2}$), and *P. falciparum* (p-value:$6.0x10^{-4}$) – although this list is likely to expand as members of the RRB family continue to be identified in these species [24].

The aforementioned studies provide strong evidence the functional clustering that is observed for both the RP and the RRB gene families represents a conserved eukaryotic genomic organizing principle. There are several potential mechanisms that could be employed to facilitate this process. One proposed mechanism involves the formation of chromosomal loops; a distinct subnuclear arrangement which would result in the clustering of promoter elements into closes physical proximity even though they are separated on the linear chromosome. A role also exists for chromatin remodeling in this process. To that end, a screen of potential chromatin remodelers identified both the Snf2 and the Chd1 complexes as necessary for coordinated expression of the *MPP10-MRX12* gene pair [23]. The influence of chromatin remodelers in the regulation of functional clusters is an attractive model to explain the non-random distribution of these clusters in many different organisms which affects so many genes. These clusters could initially form on the basis of stochastic genomic rearrangements bringing two functionally-related genes together as chromosomal neighbors. The regulatory factors and mechanisms could begin to exert an influence upon each other. Over time this confirmation is selected upon (due to the efficiency of transcriptional regulation of the pairing) and a novel functional cluster is born. Because these mechanisms are not mutually exclusive, it is likely that there are multiple layers to this processwhich contribute to the formation, maintenance, and regulation of the clusters.

Proper ribosome biosynthesis is of particular importance in a variety of organisms for several reasons: single-celled organisms tightly regulate the components necessary for the production of ribosomes to balance cellular energetic resources between growth and maintaining homeostasis with their environment. Additionally, rapid alterations to the transcriptome maximize survival and represent a selective advantage. In more complex organisms, the synthesis of the ribosome is linked to the cell-cycle and proper regulation is essential to avoid diseases such as cancers [25]. The altered expression of a single RP can be a risk factor cancer, such as *RPS19* and *RPS26* which lead to leukemia and osteosarcoma, respectively. This is also true for RRB genes such as *DKC1* and *RMRP,* which can lead to certain lymphomas, squamous carcinomas, and basal cell-carcinomas [26].The links between ribosome production and cancers explain the conservation of functional clusters in complex eukaryotes – even those that employ extensive post-transcriptional mechanisms of gene regulation.

Streamlining the Genome for the Production of Nanomachines Beyond the Ribosome

Spatial positioning of functionally-related genes into functional clusters represents an efficient way to help balance the production of proteins required in balanced, stoichiometric amounts within the cell. The exact mechanisms regulating the expression of the pairings are still an active area of study, although it has recently been found there is a degree of promiscuity for promoters and enhancers in a wide range of organisms [27]. It will be interesting to observe the extent functional clusters play in organizing the genome in other co-regulated sets of genes. A recent study set out to fully catalogue the how many functional classes of genes are found in a non-random distribution throughout the

genome. It was observed that as many as 25% of these gene families are found in a non-random distribution in budding yeast, potentially serving an organizing role in coordination of the expression of the nanomolecular machinery involved in cell wall organization, sporulation, telomere organization, lipid transport, and more [28].

Conclusion

It has long been understood that rDNA repeats cluster together in the nucleolus, streamlining expression and regulation of rRNA production. Likewise, the nonrandom genomic distribution of the RP and RRB genes throughout the genomeresulting in adjacent gene co-regulation, suggests that the spatial positioning of these genes is integral to proper transcription of both families. This is conserved in many species and functional-clustering facilitates rapid, efficient transcription and balances utilization of cellular resources appropriately. It is likely that spatial positioning of functionally-related genes may represent a fundamental, yet still under-characterized transcriptional mechanism for many functionally related gene families [28]. This arrangement streamlines the genome and potentially regulates the synthesis of a wide variety of cellular nanomachines.

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