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At the crossroads of growth control; making ribosomal RNA

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Although the mechanisms of cell cycle control are well established, the factors controlling cell growth and target size are still poorly understood. Much evidence suggests that ribosome biogenesis, and in particular the synthesis of the rRNAs, plays a central role not only in permitting growth, but also in regulating it. In the past few years we have begun to penetrate the network linking rRNA gene transcription to growth.

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Abbreviations

CE	core promoter element
CF	core factor
HDAC	histone deacetylase
NORs	nucleolar organiser regions
PolI	RNA polymerase I
RENT	regulator of nucleolar silencing and telophase exit
rRNA	ribosomal RNA
SL1	selectivity factor 1
TBP	TATA-box binding protein
TOR	target of rapamycin
UAF	upstream activating factor
UE	upstream promoter element

Introduction

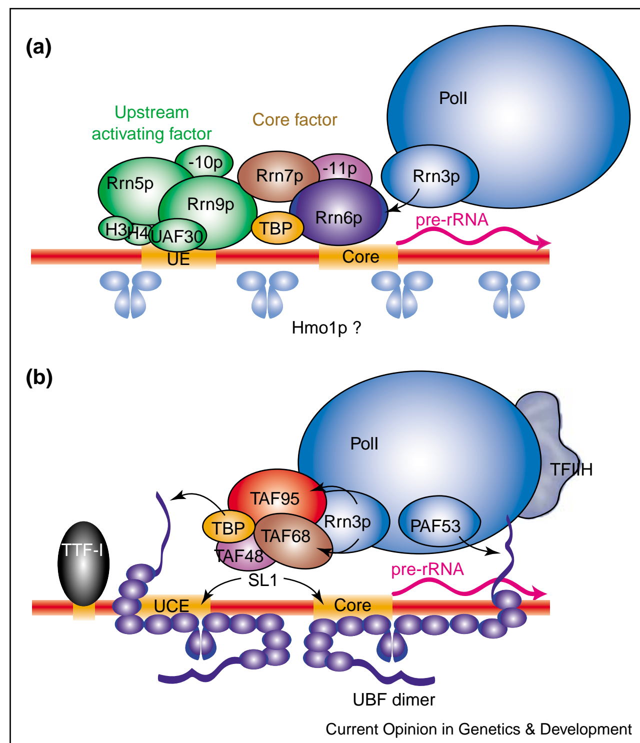
The cycle of cell growth and division is a fundamental aspect of all organisms. Its regulation permits organisms to adapt to changes in nutrients and to define the broadly varying target sizes of differentiated cells in higher eukaryotes. Its deregulation can lead to hypertrophy or to the uncontrolled proliferation characteristic of cancer. Growth control assumes a knowledge of target size, a means to detect size and a mechanism to translate this knowledge into either growth arrest or cell division. Although our understanding of the molecular switches controlling the cell cycle is well developed (e.g. see [1]), the manner in which cell size is measured and how this information is used to control the cell cycle still remains much of a mystery [2]. A central factor in determining growth is the rate of protein synthesis and hence the availability of ribosomes [3]. But 50% or more of the synthetic effort of a rapidly proliferating eukaryotic cell is expended solely in ribosome production. It is not surprising, then, that organisms strictly control ribosome biogenesis and, in particular, the synthesis of ribosomal RNA (rRNA).

The 18S, 5.8S and 28S rRNAs, the scaffold and catalytic heart of the eukaryotic ribosome [4], are transcribed as a single pre-rRNA by the dedicated RNA polymerase I (PolI). Nucleoli, the sites of ribosome biogenesis, form around clusters of rRNA gene repeats at the nucleolar organiser regions (NORs) on one or more chromosomes and their existence depends on active rRNA gene transcription [5]. This transcription is finely regulated in response to changes in growth and many environmental cues (see also [6•–8•]). In turn, the nucleoli regulate cellular functions as wide ranging as mRNA processing and mitosis [9]. For example, mitotic exit is controlled by nucleolar release of Cdc14p, a component of the RENT (regulator of nucleolar silencing and telophase exit) complex [10]. Arrest of cell-cycle progression by the tumour suppressor p53 also falls under nucleolar control [11,12]. Thus, the rRNA genes form a key component of the signaling network controlling cell growth and proliferation. In turn, growth regulation of the rRNA genes is surprisingly rapid, even placing them in the immediate response category along with several oncogenes [13••]. Here, I review the mechanisms underlying this regulation.

The ‘basals’ of PolI transcription

In yeast, two basal factors, upstream activating factor (UAF), associated with the TATA-box binding protein (TBP), and core factor (CF), respectively, bind the upstream and core promoter elements (UE and CE, respectively) [5,6•] (Figure 1a). Similarly, two mammalian basal transcription factors have been identified, the so-called upstream binding factor (UBF), a multiple-HMG1-box protein, and selectivity factor 1 (SL1), containing TBP and three associated factors, $TAF_I95/110$, 68/63 and 48 [6•–8•] (Figure 1b). Rrn7p and 11p of the yeast CF bear some homology respectively to TAF_I 68 and 48, suggesting an equivalence of CF and SL1 [14]. But, in yeast TBP is purified in association with UAF not with CF, although TBP may form a common subunit in a UAF–CF supercomplex [15,16]. UAF is stably associated with the UE whereas CF binding appears cyclic [17,18]. Hence, disruption of the UAF–UE interaction causes promoter inactivation. As yet, recombinant forms of mammalian SL1 are insufficient for transcription [19]. Thus, the homologies between the mammalian and yeast TAFs and the apparent higher complexity of the yeast system

Figure 1



The PolI initiation complex. **(a)** The situation in yeast and **(b)** in mammals. The various polypeptides that have been associated with UAF, CF, SL1 and PolI are indicated as ellipsoids. The individual HMG1boxes of UBF are also shown as ellipsoids. Common colours indicate potential homologs among yeast and mammalian TAFs.

all suggest that a mammalian UAF-like complex must still be found. The PolII basal and transcription coupled repair factor TFIIH may also play a role in PolI transcription [20,21]. However, doubt still exists as to whether TFIIH loss affects PolI transcription directly or via the transcription of PolII genes [22].

In mammals, it is believed that UBF aids SL1 in its ability to recognise the promoter, possibly via DNA looping [23]. UBF binds throughout the PolI promoter via at least three of its HMG1-boxes, which bind and kink the DNA. However, a post-initiation role for UBF has also been demonstrated [24]. Hmo1p, a single HMG1-box nucleolar protein vaguely resembling UBF, strongly enhances rRNA gene transcription and cell growth in yeast [25]. Both UBF and Hmo1 belong to the sequence non-specific class of HMG1-box proteins [26]. Consistent with this and with its high abundance, UBF is found bound throughout the rRNA genes [27••]. Thus, both UBF and HmoIp may define an rRNA gene-specific chromatin.

Gene activation — regulation by numbers?

Typically 100s to 1000s of copies of the rRNA genes exist as tandem repeats at either one or several NORs. Regulation of rRNA gene transcription could then logically occur by modulating the activity of the transcription machinery, by changing the number of active genes or both. But, although changes in the number of active genes have been observed, several lines of evidence suggest that they are in fact not important in regulating pre-rRNA transcription rates. Differential accessibility of the rRNA genes to the DNA crosslinker psoralen led to the surprising conclusion that, in both higher and lower eukaryotes, no more than ~50% of chromosomal ribosomal genes are active at any given time [28••,29]. Consistent with this, both yeast and *Drosophila* require less than half of their normal rRNA gene complement to survive [30,31]. On the other hand, in yeast cells carrying the minimum viable number of rRNA genes, most if not all genes are transcribed [32]. When grown into stationary phase, yeast reduces pre-rRNA synthesis by 10 or more times. Concomitantly it also reduces the proportion of its rRNA genes that are actively transcribed [33••]. Modification of chromatin has become a key theme in our understanding of gene regulation [34]. Thus, it was not surprising to find that loss of histone acetylation, in this case at lysines (K) -5 and -12 of H4, was correlated with yeast rRNA gene inactivation. This data implicates the histone deacetylase Rpd3p (homolog of HDAC1,2) and perhaps the opposing acetyl-transferase Esa1p (TIP60) [35••]. However, although loss of Rpd3p does prevent inactivation of rRNA genes, it does not prevent the normal down-regulation of transcription associated with stationary phase [33••]. Thus, although H4–K5 and –K12 deacetylation correlates with gene inactivation and Rpd3 is essential for this inactivation, neither are necessary to down-regulate transcription in stationary phase. A more rapid 5–10 times down-regulation of yeast rRNA gene transcription is observed when the TOR (target of rapamycin) nutrient sensing pathway is inactivated [2]. This down-regulation is also accompanied by a deacetylation of H4–K5 and -K12 [35••]. However, whereas rapamycin down-regulation of pre-rRNA synthesis was claimed to be Rpd3p-dependent in one study [35••], in another it was not accompanied by gene inactivation, the apparent function of Rpd3 [36••]. Left out of this picture is the role of the UBF-like small HMG-box protein HmoI. Mammalian UBF activity is, in fact, directly regulated by an acetylation–deacetylation cycle implicating acetyl-transferase CBP and deacetylase HDAC1 [37]. Could then the key target of Rpd3p be HmoI and not H4? Whatever the mechanism of gene inactivation in yeast, it does not appear to play a role in down-regulating the global level of pre-rRNA gene transcription.

Silencing in yeast — keeping PolI on but PolII off

Gene 'silencing' at the yeast NOR refers not to the inactivation of rRNA genes but rather to the suppression of recombination, to the inactivation of PolII genes transposed into the NOR and to the inhibition of PolII

transcription of the rRNA genes themselves [38,39]. PolII silencing of the yeast NOR requires the NAD-dependent deacetylase Sir2p, the PolI promoter binding factor UAF (Figure 1) and is unidirectionally spread along the locus by active PolI transcription [40••,41••]. Sir2p, a regulator of aging and part of the nucleolar RENT complex bound to PolI [42••], is responsible for deacetylating K16 of H4 and K9,14 of H3 at the yeast NOR [42••,43,44], events essential for heterochromatin formation in eukaryotes. Loss of Rpd3p enhances yeast silencing in a Sir2p-dependent manner [45–47]. Thus, enhanced Esa1 acetylation H4–K5 and -K12 on the rRNA genes, one result of Rpd3p loss, is associated with more effective exclusion of PolII, but, as noted above, with the inability to prevent PolI transcription of these genes. Perhaps, increased Esa1 histone acetylation throughout the genome tightens the borders of heterochromatin making more Sir2p available for NOR silencing (e.g. see [48]). Alternatively, Rpd3p loss might increase PolI loading on the active fraction of rRNA genes, two-fold seems possible [32], physically excluding PolII and at the same time augmenting Sir2p recruitment. Thus, it seems that Rpd3p provides the means to switch individual rRNA genes off, whereas Sir2p either helps in excluding PolII from the active rRNA genes or perhaps primes them such that when they are switched off they are inaccessible to PolII. (Remember, at any moment ~50% of rRNA genes are not transcribed by PolI. Some of these genes may still be accessible to PolII [40••].) The rRNA genes may then be maintained in a PolI accessible but PolII inaccessible 'heterochromatin' configuration by the combined actions of Esa1p acetylation of H4–K5,12 and Sir2p deacetylation of H4–K16 and H3–K9,14. Additionally, Set1p methylation of H3–K4 that normally correlates with transcriptional activity of mRNA genes, actually enhances PolII silencing in the rRNA genes, although it is partially antagonised by Sir2p silencing [49•,50•]. Clearly, a mechanistic interpretation of these data must await further studies. But it is tempting to suggest that PolI and PolII transcription may be affected quite differently by any given combination of chromatin modifications.

Constitutive silencing — keeping PolI out

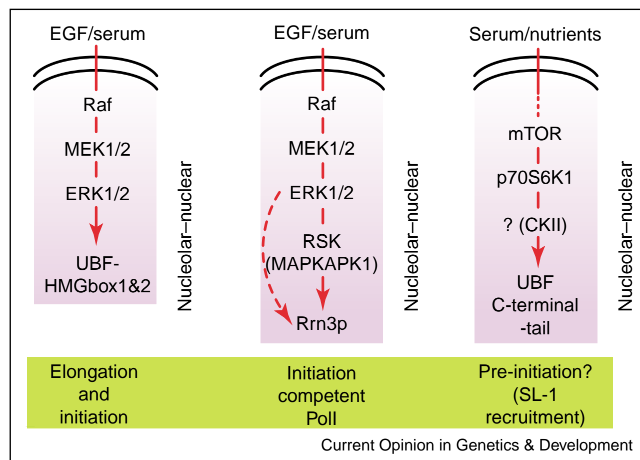
Although Rpd3p deacetylase is responsible for rRNA gene inactivation in the stationary growth phase in yeast, loss of Rpd3p does not lead to the re-activation of the 50% or so of silent genes [33••]. Thus, at least two distinct states of rRNA gene inactivation exist in yeast, a facultative one mediated by Rpd3p and a constitutive one of unknown origin. As yet, only a long-term constitutive silencing of the rRNA genes, referred to genetically as nucleolar dominance, has been recognised in higher eukaryotes. It is common in plants and animals and is recognised when NOR-carrying chromosomes of different species are present in the same cell [51–53]. In these cases, silencing is of a complete NOR, but whether this type of constitutive silencing is responsible for maintaining 50% of the rRNA genes inactive at any given moment is still far from clear. In some cases, DNA methylation has been suggested to be responsible [53,54], but in other cases methylation *per se* is not the answer [55]. Naturally occurring cytosine methylation in the UCE of the mouse PolI promoter (Figure 1) has been shown to modify, but not eliminate, binding of UBF and in this way may prevent transcription [56]. Promoter proximal recruitment of a DNA methyltransferase and a SIN3-like complex, including HDAC1, via the chromatin-remodeling complex NoRC and the PolI termination factor TTF-I (Figure 1) was shown to inactivate a reporter gene in a DNA methylation dependent manner [57]. This inactivation of PolI transcription was also associated with H3–K9 methylation [58••]. H3–K9 methylation has been correlated with PolII silencing in higher eukaryotes but does not occur in either yeast or tetrahymena (e.g. see [49•,50•]). However, these data fly in the face of earlier reports that termination and the promoter proximal termination site are essential to promoter activity [59–62].

Clearly we are still quite some way from understanding rRNA gene inactivation, but it seems that in higher eukaryotes three distinct rRNA gene inactivation states may co-exist. The first two probably equate with facultative and constitutive silencing in yeast whereas the last is a more stable silencing characterised by nucleolar dominance. As no change in the number of actively transcribed rRNA genes has ever been documented in higher eukaryotes [29], none of these states apparently influence the levels of pre-rRNA synthesis. Underlining this, we recently observed that five-fold changes in pre-rRNA synthesis in both human and mouse cells can be induced in the complete absence of a change in actively transcribed gene number (V Stefanovsky, T Moss, unpublished). Yet if gene inactivation and silencing play no apparent role in transcription, why do they occur? Possibly they occur to enhance the efficiency of ribosome assembly by concentrating it around fewer active genes. They might also better prevent ingression of PolII, which is presumably detrimental to normal ribosome biogenesis, or stabilise the rRNA gene repeats by repressing recombination, though these appear less likely [40••]. Limiting the number of transcribed genes might also limit DNA damage, perhaps creating a somatic rRNA gene 'germline' (e.g. see [6•,63]).

Getting PolI going, and keeping it going

Consistent with the active gene number playing either little or no role in pre-rRNA transcription rates, neither the yeast UAF/CF nor mammalian SL1 complexes have been found to be growth regulated. SL1 is, however, inactivated during mitosis by CDK1 phosphorylation and the tumour suppressors p53 and Rb were shown to prevent recruitment of SL1 by UBF [64–66]. UBF activity is also regulated during the cell cycle by CDK2/4 [67] and is further regulated by competitive CBP/Rb–HDAC

Figure 2



Summary of the regulatory pathways controlling rRNA gene transcription that have been established to date. The intracellular signal transduction molecules that have been implicated in each pathway and the transcription factor target are indicated. Below each pathway, the probable mechanism of rRNA gene regulation is also indicated. CKII, casein kinase II.

binding and by acetylation/deacetylation [37]. Though these regulatory mechanisms demonstrate a parallel control of rRNA gene transcription and cell proliferation, their relation to growth regulation is less clear. The first molecular links with growth came when UBF was shown to be controlled by two major growth-regulatory pathways, the ERK1/2 MAP-kinase cascade and the TOR cascade (Figure 2). Two of the six HMG1-boxes of UBF were found to be phosphorylated directly by ERK1/2 MAP-kinases and these phosphorylation events were shown to be essential for increased rRNA gene transcription [13••]. At the time, it was postulated that ERK regulated transcription at a post-initiation step. Consistent with contemporary work [24], we now know that unphosphorylated UBF blocks early PolI elongation, whereas ERK-phosphorylated UBF enhances the elongation process (V Stefanovsky, T Moss, unpublished). The TOR pathway has also been shown to activate UBF via a p70S6 kinase dependent phosphorylation of its C-terminal tail [68••]. As phosphorylation of the UBF tail was shown to enhance recruitment of SL1 [69], the TOR pathway may predominantly regulate pre-initiation complex formation. Not surprisingly, absolute UBF levels are limiting for PolI transcription [70] and their regulation by growth stimuli have been reported [71]. Myc oncogene enhances rRNA gene transcription by increasing UBF levels (R Hannan, personal communication), but it may also act more directly (R White, personal communication).

Switching PolI on and off

Over 20 years ago, growth regulation was associated with a specific form of PolI [72–75]; but a molecular explanation has only recently begun to evolve. PolI is found in two forms, both synthetically active, but only one, variously called Pol IB, Factor C, Pol Iβ, is able to specifically initiate pre-rRNA transcripts. The essential yeast factor Rrn3p [76] and its mammalian homologs (Rrn3 and TIF-IA) [77,78] are found associated with the initiation-competent form of PolI [78–81]. Soon after initiation, Rrn3p is released in an inactive dephosphorylated state and probably recycled after rephosphorylation [36••,79,82•,83]. Thus, Rrn3p resembles TFIIF of PolII and σ of eubacterial polymerase [84,85]. However, unlike either TFIIF or σ, Rrn3p may not be required for PolI to recognise and bind the promoter [17].

Rrn3 is inactivated in both yeast and mammals in response to inhibition of protein synthesis, growth-limiting conditions and extracellular signaling [79,82•,86]. EGF, serum and TPA stimulation correlate with MEK1/2-ERK1 dependent C-terminal phosphorylation of mouse Rrn3, probably by RSK (p90S6 kinase or MAPKAP kinase1), and mutation of the phosphorylation sites prevents transcription [86] (Figure 2). However, RSK activation is not generally correlated with growth and in one system even leads to cell cycle arrest associated with the complete inhibition of the rRNA genes [87]. The activity of mouse Rrn3 may also be down-regulated by TOR inactivation, possibly via p70S6 kinase inactivation (I Grummt, personal communication), but others did not observe such a regulation [68••].

It has been asserted that PolI initiation rates in mouse cells are solely regulated by the active levels of Rrn3 (TIF-IA) [86]. Although this may be true for yeast [36••], it conflicts with data showing regulation via UBF in mammals. If initiation rates were only regulated via Rrn3, pre-rRNA synthesis rates should be directly proportional to the number of polymerases engaged on the rRNA genes. We tested this prediction in three human and mouse cell lines and found that five-fold changes in transcription rate occur in the near absence of changes in the numbers of polymerases engaged on the rRNA genes [13••] (T Gagnon-Kugler, T Moss, unpublished). Thus, PolI elongation rates, not PolI initiation rates, are the limiting factor in these cells. This is consistent with the proposed post-initiation role for UBF discussed above. Further, Rrn3p is not the only PolI-associated factor to be regulated in response to growth changes. Rat TFIC, originally found as a PolI associated dexamethasone inhibited factor in a rat lymphosarcoma cell line and as yet not identified at the molecular level, has been shown to be distinct from Rrn3 [82•].

Process or destroy

Recent studies of proteins associated with the regulation of the p53 tumour suppressor have revealed some quite unexpected results. The tumour suppressor p14ARF, which in conjunction with Mdm2 regulates p53 levels,

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was found to suppress processing of pre-rRNA by inactivating B23, a nuclease that carries out early pre-rRNA cleavage [88,89]. This in itself was very exciting because it showed that a tumour suppressor could modulate rRNA production post-transcriptionally. But because inhibition of pre-rRNA processing did not lead to increased pre-rRNA levels, the data further suggest that degradation may be another important factor in regulating rRNA production.

Conclusions

Factors affecting growth and proliferation in yeast and mammals impinge both directly and rapidly on the transcription of the rRNA genes. However, it would appear that rRNA synthesis is not significantly affected by changes in active gene number, although this could play a role in regulating nucleolar structure and ribosome biogenesis. Rather, regulation occurs at the levels of transcript initiation, elongation, processing and degradation via a rich combination of mechanisms. Future work must now attempt to understand how these mechanisms are coordinated and decide whether the rRNA genes are permissive or active players in growth regulation.

Update

Strohner *et al.* [90] have now defined more precisely the role of NoRC in silencing the mouse PolI promoter. They suggest that the formation of chromatin *per se* is insufficient to silence the promoter and that recruitment of NoRC via TTF-I induces a chromatin-remodeling step essential for silencing [1]. This, to some extent, conflicts with these authors' previous demontration that TTF-I binding upstream of the promoter (Figure 1) relieves chromatin-mediated transcription inactivation in mouse.

Acknowledgements

This review has attempted to put into context recent work of some direct relevance to the growth regulation of rRNA gene transcription. For this reason and due to constraints on the length of the review, reference to much very important work on the mechanisms of PolI transcription has had to be omitted and I wish to apologise to those whose work has apparently been overlooked. I would also like to thank those who provided updates on their most recent unpublished work and those who took the time to discuss various mechanistic scenarios with me. Finally, I wish to acknowledge J Coté, V Stefanovsky, T Gagnon-Kugler, F Langlois and N Bisson for critical discussions and their help in preparing this review. This work was supported in Canada by an operating grant from the Canadian Institutes of Health Research (CIHR). The University Laval Cancer Research Centre is supported by the Fonds de Recherche sur la Santé du Québec (FRSQ).

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