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Review

One step back before moving forward: Regulation of transcription elongation by arrest and backtracking

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ABSTRACT

RNA polymerase II backtracking is a well-known phenomenon, but its involvement in gene regulation is yet to be addressed. Structural studies into the backtracked complex, new reactivation mechanisms and genome-wide approaches are shedding some light on this interesting aspect of gene transcription. In this review, we briefly summarise these new findings, comment about some results recently obtained in our laboratory, and propose a new model for the influence of the chromatin context on RNA polymerase II backtracking.

1. Introduction

RNA polymerase II plays a key role in the expression of eukaryotic genomes. All protein-encoding genes are transcribed by this polymerase, whose structural and functional properties enable the great diversity of regulatory programs that are executed throughout the genome. The best-known regulation mechanisms operate during pre-initiation complex assembly and initiation, but a significant number of genes regulate their transcription in post-initiation steps (reviewed in [1]). Among others, the mechanisms sustaining elongation should provide the opportunity to regulate transcription.

Transcription elongation is far from a uniform process. RNA polymerase II profiles, measured by chromatin immunoprecipitation (ChIP), transcriptional run-on or nascent RNA sequencing have established clear differences between genes within the same genome [2–5]. Single-cell experiments have also shown that RNA polymerase II progression along a transcribed gene is a discontinuous process which combines short advances with pauses of a variable time extent [6]. All these results demonstrate that pausing is a frequent phenomenon that is likely consubstantial to RNA polymerase II-dependent transcription.

In vitro experiments have demonstrated that RNA polymerase II pausing is a highly unstable state which results in either forward transcription or stable arrest [7]. RNA polymerase II arrest involves backtracking, a reverse movement that brings about loss of contact between the 3′ end of nascent RNA and the RNA polymerase II active site. As a result of this mislocalisation, backtracked RNA polymerases cannot resume transcription immediately. The ternary complex formed by DNA, RNA and backtracked RNA polymerase II is extremely stable. Recent structural data published by Cramer's lab explain this stability by the specific binding of eight nucleotides of backtracked RNA to a highly conserved site in the RNA polymerase II pore and funnel, which traps a protein loop located in the active site [8].

In vivo and in vitro studies have found a large number of causes which bring about RNA polymerase II arrest. The scarcity of nucleotides favours RNA polymerase II arrest in vitro. Drugs that provoke depletion of NTP pools, like 6-azauracil and mycophenolic acid, lower the elongation rate and the processivity of RNA polymerase II in vivo [9], which increases frequency of arrest [10]. Yeast mutants lacking RNA polymerase II reactivation factors, like TFIIS or Ccr4-Not (see later), exhibit hypersensitivity to these NTP-depleting drugs.

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A very intuitive cause of RNA polymerase II arrest is the presence of obstacles in DNA. Among them, nucleosomes are the most ubiquitous structures to hinder eukaryotic transcription. In vitro studies have clearly shown that nucleosomes promote backtracking and that transcription of nucleosomal templates is stimulated by TFIIS, this being the RNA cleavage factor that reactivates backtracked RNA polymerase II [11](see later).

Transcription elongation generates torsional stress on DNA, which needs to be removed by toposiomerases [12]. This accumulation of positive supercoiling is particularly detrimental for transcription elongation through yeast genes longer than 3 kpb [13]. Topological constrains is, therefore, another potential source of RNA polymerase II stalling. Interestingly, the impact of DNA torsional stress on transcription is conditioned by the chromatin organisation of the transcription unit, in a double way. On one hand, chromatin compaction facilitates RNA polymerase II elongation [14]; on the other hand, nucleosome free regions facilitate topoisomerase II recruitment to transcribed genes [15].

DNA lesions are cause of RNA polymerase arrest as well. Bulky DNA adducts within transcribed regions lead to RNA polymerase II arrest. In most cases this arrest is irreversible and needs to be solved by the degradation of the stalled polymerase (reviewed in [16]). Undamaged DNA sequences can also favour RNA polymerase II arrest in vitro, even when the template takes a non-nucleosomal configuration [17]. Pyrimidine-rich tracks in the non-template strand, like the TTTTTTTCTCCATTTT sequence present in the intrinsic "terminator" region within the c-*myc* first exon–intron boundary, induce RNA polymerase II arrest [18]. This sequence-dependent arrest was first explained by the results of the weak DNA–RNA interactions caused by the high proportion of U = A pairs. Later, however, results indicate that the presence of cytosines in the polypyrimidine track is not detrimental for arrest [19]. This is in good agreement with the strong preference for pyrimidines found in the specific interaction between backtracked RNA and the "backtracked site", in the RNA polymerase II pore and funnel [8]. This sequence specificity establishes the basis for an arresting code across the genome, which might explain the biased distribution of the polypyrimidine/polypurine tracks between template and non-template strands [20].

2. Molecular mechanisms overcoming RNA polymerase II arrest

RNA polymerase arrest cannot be permanent, since this would involve the complete repression of gene expression and would seriously hinder DNA replication, leading to genome instability and eventually to cell death (reviewed in [16]). Even in those cases where RNA polymerase arrest plays a role in gene regulation, the mechanisms capable of resuming transcription or removing arrested polymerase are required for transcription cycle viability. Three different mechanisms operating in arrested RNA polymerase II have been described: RNA cleavage, allowing the RNA 3′ end to relocate at the active site; reversion of the backtracked state by the forward movement of the polymerase; and eviction of arrested RNA polymerase II by ubiquitylation-mediated degradation (Fig. 1).

Endonucleolytic activity allows 3′ RNA cleavage, which is an inherent property of RNA polymerase II that becomes highly stimulated by cleavage factor TFIIS [21]. The structural basis for TFIIS-assisted RNA cleavage has been recently explained [8]. TFIIS domain III extends into the RNA polymerase II pore, reaching the active site and remodels the RNA polymerase II motifs that bind backtracked RNA. This enables the displacement of RNA from the backtracked site. In addition, TFIIS complements the active site with several residues that may catalyze proton transfers during RNA cleavage [8].

Even in the absence of TFIIS, arrested RNA polymerase II can be reactivated by RNA cleavage. This has been elegantly shown by Svesjtrup's lab using a dominant negative TFIIS mutant that can bind arrested RNA polymerase II without stimulating RNA cleavage. The resulting ternary complex stabilises the backtracked configuration by inhibiting spontaneous RNA cleavage [22]. The lethal phenotype of this mutant in yeast, unlike the viability of the TFIIS deletion mutant, indicates that RNA polymerase II arrest is a very common phenomenon which yeast cells under standard growing conditions can solve by either spontaneous or TFIIS-stimulated RNA cleavage, or even by alternative mechanisms.

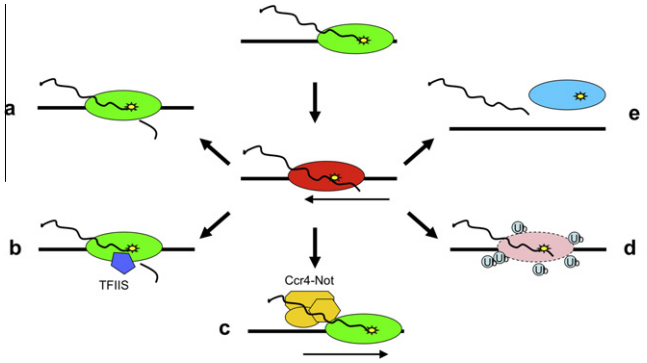

Fig. 1. Mechanisms resolving RNA polymerase II backtracking. RNA polymerase II backtracking involves the relocation of the 3′ end of nascent RNA outside the active site. This can be solved spontaneously by the intrinsic RNA cleavage activity of the enzyme (a) but it gets strongly stimulated by TFIIS (b). Alternatively Ccr4, a subunit of the Ccr4-Not complex, can stimulate forward-tracking, thus allowing RNA polymerase II to resume elongation in an RNA cleavage-independent manner (c). Arrested RNA polymerase II can be removed from DNA by ubiquitylation and its subsequent degradation by the proteasome (d). The ternary complex formed by DNA, RNA and the backtracked RNA polymerases might also be disassembled, involving transcription termination (e). However no clear experimental evidence is available for this mechanism.

One of these alternative mechanisms is mediated by Ccr4-Not. This is an evolutionarily conserved complex composed of nine subunits. Based on independent experimental approaches, Ccr4-Not has been connected to different aspects of gene transcription, including initiation [23,24] and elongation [2,25,26]. Ccr4 is the major cytoplasmic mRNA deadenylase in yeast and the complex localises to processing bodies where mRNA degradation takes place [27,28]. In this multifunctionality context, Reese's lab has demonstrated that Ccr4-Not stimulates transcription elongation by promoting the resumption of elongation by arrested polymerases [29]. The interaction of Ccr4-Not with the emerging transcript is required for this way of reactivating arrested RNA polymerase II, which does not involve RNA cleavage (Fig. 1).

The described reactivation mechanisms are likely to overcome most RNA polymerase II arrest events, but cells have an additional way of contending with irreversibly blocked transcriptional complexes, namely degradation [30,31]. This mechanism involves ubiquitylation of the biggest RNA polymerase II subunit, Rpb1, at different sites in a process that is specifically mediated by the ubiquitylation factor Def1 and the CTD domain of Rpb1 [32]. Mutations affecting Rpb1 ubiquitylation and RNA cleavage are synthetic lethal, indicating that RNA cleavage or degradation are the only possible alternatives, at least for a subset of arrested RNA polymerase II molecules [32]. In addition to the proteasome-dependent degradation of RNA polymerase II, Rpb1 ubiquitylation might also promote other alternative resumption processes prior to degradation (see the discussion of this issue in [33]). In light of this, it is meaningful that Def1 is an interacting factor of Rad26, a DNA helicase involved in transcription-couple repair, which inhibits RNA polymerase II degradation [34]. A scenario, in which Rpb1 ubiquitylation promotes the disassembly of arrested RNA polymerase II in a helicase-assisted way, is an attractive possibility that remains to be experimentally tested (Fig. 1).

3. Gene regulation by RNA polymerase II arrest

Transcriptional regulation in post-initiation stages is a common phenomenon across metazoan genomes. At least 40% of genes show promoter-proximal accumulation of RNA polymerase II in mammalian, Drosophila and Caenorhabditis cells [3,35–39].

An example of regulation at the level of elongation is found in the so-called bivalent genes in embryonic stem cells. They display characteristic chromatin markers of silenced (histone H3K27 tri-methylation) and active transcription (histone H3K4 tri-methylation)[40]. A subgroup of bivalent genes, bound by Polycomb repressive complex 2, displays stalled RNA polymerase II. Since stable pausing leads to RNA polymerase II arrest, it is likely that the RNA polymerase II molecules present in silenced bivalent genes are arrested; however, there is no direct experimental evidence for this hypothesis. Such evidence actually exists for another group of mammalian genes which exhibit promoter-proximal accumulation of RNA polymerase II. Growth-promoting, pro-oncogenic genes like *FOS* and *MYC* reside preferentially in compact chromatin and are inefficiently transcribed under basal conditions. The transcription of these genes is repressed during early elongation by a mechanism interfering with TFIIS recruitment that involves H2B ubiquitylation [41].

The involvement of TFIIS is also demonstrated in the activation, in response to heat shock, of the stalled RNA polymerases occupying the promoter proximal region of Drosophila Hsp70 [42]. Using RNA interference, in vivo TFIIS depletion provokes a delay in Hsp70 induction which, in this case, is only possible after a new round of transcription initiation [42]. Accordingly, we can conclude that most, if not all, stably stalled RNA polymerases would be backtracked and would require TFIIS or any alternative reactivation mechanism to recover the elongation capability upon activation.

One direct way of detecting backtracked RNA polymerases is to compare the genomic patterns of run-on signals, which reflect elongation-proficient RNA polymerases [43], with the genomic distribution of the total RNA polymerase II measured by ChIP. This strategy detected clear differences between the two patterns across the yeast genome [44]. There is a general high correlation between run-on and ChIP signals, but some gene ontology categories exhibit lower run-on values than expected according to their ChIP signals [44]. This difference proves particularly striking for those genes encoding ribosomal proteins (RP), indicating the accumulation of backtracked RNA polymerase II in these genes. One particular behaviour of RNA polymerase II during transcription elongation of RP genes is also reflected by the 5′-biased distribution of the run-on signals in this specific group of genes [2]. This bias was also observed in the distribution of nascent RNA of highly expressed genes across the yeast genome [5]; the high proportion of highly transcribed genes belonging to the RP group might explain this result.

The run-on signals of RP genes lowered when comparing yeast cells exponentially growing in glucose-containing medium to cells exponentially growing in galactose-containing medium. However, this decrease was significantly more marked in the total RNA polymerase II levels detected by ChIP than in the run-on signals; accordingly, the run-on/ChIP ratio for the RP genes increased from glucose to galactose (Fig. 2). The nuclear genes encoding mitochondrial components exhibited the opposite change (Fig. 2) [44]. The simplest interpretation of these results involves a specific reduction in the number of backtracked RNA polymerase II complexes in response to the carbon source (Fig. 2). In fact, the run-on/ChIP ratio of RP genes depends on the integrity of the Ras-PKA pathway and on the silencing domain of Rap1, an essential transcription factor for RP genes transcription [44]. In short, these observations suggest that RNA polymerase II backtracking is an important element in the transcriptional regulation of RP genes and of other gene families across the yeast genome.

A regulatory mechanism based on RNA polymerase II backtracking, for constitutively expressed genes like RP, must involve an efficient reactivation mechanism. A series of experiments carried out in our laboratory demonstrate that TFIIS is particularly involved in the reactivation of backtracked RNA polymerase II on RP genes, at least under the strong transcriptional stress caused by 6AU [45]. Shortly after adding this drug to yeast cells, RNA polymerase II complexes transcribing the RP genes become enriched in TFIIS, in comparison to the other highly transcribed genes tested, which do not relate to ribosomes (Fig. 3A). This difference between RP and non-RP genes is particularly striking in the 5′ end of the transcribed region, where RNA polymerase II accumulates upon NTP depletion [9]. RP genes maintain high TFIIS/Rpb3 ratios in 5′ upon 6AU treatment, whereas in non-RP genes RNA polymerase II shifts towards the 5′ region while TFIIS does not, resulting in low TFIIS/Rpb3 ratios (Fig. 3A). The low TFIIS demand by paused RNA polymerase II in non-RP genes anticipates a lower level of backtracking than in RP genes, which is confirmed by the transcriptional run-on assays (Fig. 3B). In fact, the comparison of wild-type and *dst1Δ* (TFIIS-deleted) strains shows that the run-on/RNA polymerase II ChIP ratios of RP genes in the presence of 6AU are strictly dependent on TFIIS, whereas the effect of 6AU on non-RP genes' run-on/RNA polymerase ChIP ratios is much milder and does not depend on TFIIS to a similar extend (Fig. 3B).

We have also showed that this high dependence of yeast RP gene transcription on TFIIS can be suppressed by deleting genes encoding transcriptional regulators of RP gene expression, like Sfp1 [45]. In the absence of Sfp1, RP genes do not depend on TFIIS to maintain active elongating RNA polymerase molecules on their gene bodies (Fig. 3B). Moreover, the absence of Sfp1 suppresses RNA polymerase II accumulation on the 5′ end in all tested genes, suggesting that this protein plays a general role in promoting RNA polymerase II pausing [45].

By way of conclusion, the specific regulation imposed by factors like Sfp1 to yeast RP genes during elongation, involving RNA polymerase II backtracking, makes this group of genes more dependent on reactivation factors, like TFIIS, to resist transcriptional stress.

4. Backtracking in the chromatin context

The mechanism explaining this differential tendency of RNA polymerase II to backtrack is unknown. It may be due to a differential composition of RNA polymerase II elongation machinery, thereby imposing different modes of transcription to specific genes or regulons. A biased composition of RNA polymerase II itself is an appealing hypothesis. The Rpb4/7 submodule has been shown to drop easily from RNA polymerase II in vitro and to play independent roles in vivo [46]. *rpb4Δ* mutants display synthetic lethality with *dst1Δ*, lacking TFIIS, and are extremely sensitive to NTP-depleting drugs [26,47]. It would be, therefore, conceivable that in some genes RNA pol II was prone to backtracking as a result of a premature drop of Rpb4/7 during elongation. Experimental data, however, do not support this hypothesis so far. The Rpb3/Rpb7 ratio is largely invariable across the yeast genome [48]. Moreover, the composition of the whole RNA polymerase elongation machinery is largely constant for all yeast genes [49], suggesting that the differential backtracking behaviour is likely due to transcribed genes rather than to transcriptional machinery.

In contrast, chromatin is markedly polymorphic across the genome and nucleosome positioning is not uniform. Yeast RP genes, for instance, show shorter nucleosome repeat than non-RP genes [50]. Histone variants and chromatin covalent modifications also display gene-to-gene variation across the genome [51]. Accord-

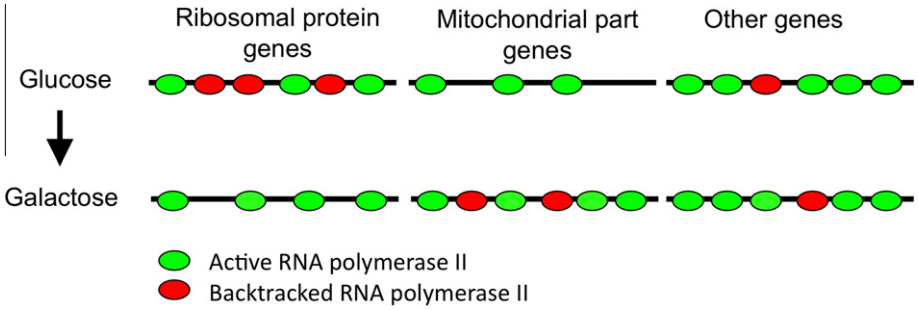

Fig. 2. The proportion of active, run-on-competent RNA polymerase II is regulon-specific and controlled by the yeast cell in response to physiological stimuli. Yeast ribosomal protein genes show a significant proportion of elongating RNA polymerase II molecules that do not produce a transcriptional run-on signal (red) when exponentially growing in glucose-containing medium. The same genes, when cells are exponentially growing in galactose-containing medium, show lower levels of elongating RNA polymerase II but exhibit a much higher proportion of run-on competent enzymes (green). The yeast genes encoding mitochondrial elements exhibit the opposite regulation: low proportion of active polymerase in galactose medium and high proportion, although limited levels, in glucose medium. Most of yeast genes do not exhibit a significant proportion of run-on-incompetent cells under either of the two growth conditions. Adapted from the experimental data described in [44].

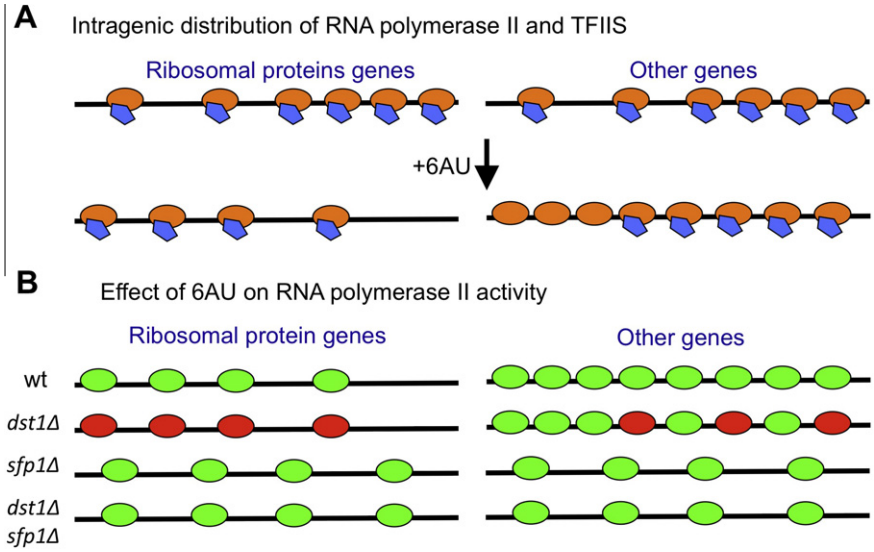

Fig. 3. TFIIS is required particularly to maintain RNA polymerase II activity on ribosomal protein genes under transcriptional stress. A. A 15 min 6AU treatment causes a 5′-shift in the distribution of RNA polymerase II along the transcribed region in wild-type yeast cells. At the same time, RP genes decrease at the absolute levels of RNA polymerase II, which reflects their down-regulation in response to the growth impairment caused by the drug. TFIIS distribution parallels that of RNA polymerase II in RP genes. In non-RP genes, TFIIS does not undergo the 5′-shift of RNA polymerase II upon 6AU treatment. **B.** Comparison of the wild-type and *dst1Δ* run-on patterns allow us to conclude that RP genes require TFIIS to maintain the activity of their RNA polymerase molecules upon 6AU addition. Non-RP genes show a much milder dependence on TFIIS under the same conditions. This differential behaviour of RP and non-RP genes depends on regulatory factor Sfp1. In both *sfp1Δ* and *sfp1Δ dst1Δ* cells, RNA polymerase activity and intragenic distribution are not influenced by 6AU. Adapted from the experimental data described in [45].

ingly, the differential functional interaction between RNA polymerase II and the gene-specific configuration of chromatin might explain its differential tendency to backtrack. If this hypothesis were true, elongation through some chromatin configurations would take place without backtracking, whereas other alternative chromatin configurations would be highly prone to backtracking. In the latter, TFIIS would be highly demanded. In vitro experiments performed in Kashlev's laboratory have shown that TFIIS is indeed required for RNA polymerase II to overcome the strong stalling imposed by a nucleosome, whereas the same DNA template, when naked, does not promote RNA polymerase II pausing or requires TFIIS [52]. The combination of TFIIS and TFIIF, another general transcription factor that stimulates elongation, synergistically stimulate nucleosome transcription by RNA polymerase II [53].

In these latter experiments, nucleosome traversal by RNA polymerase II took place without nucleosome displacement. Studitsky's laboratory has shown that nucleosomes can indeed survive transcription in vitro, by allowing the formation of an intranucleosomal DNA loop (Ø loop) that contains the transcribing enzyme [54]. This Ø loop likely requires specific DNA-histone interactions, since histone Sin mutations, disrupting this kind of interactions, compromise nucleosome survival during elongation [55].

Recent results obtained by atomic force microscopy also indicate that RNA polymerase II can transcribe a nucleosome without promoting its complete disassembly, but just removing a single H2A-H2B dimer [56]. A similar conclusion was obtained by analysing the fate of nucleosomal histones during in vitro transcription: a remodelled nucleosome, depleted of a single H2A-H2B dimer by histone chaperone Nap1 action, can be transcribed without complete histone eviction [57]. In agreement with these results, histone hexasomes (a H3-H4 tetramer associated to a single H2A-H2B dimer in the context of a remodelled nucleosome [58]) have long since been related to transcribed chromatin [59].

In this emerging model, RNA polymerase II would be able to elongate transcription along a nucleosomal DNA template by taking advantage of the Ø loop that the missing H2A-H2B dimer would provoke within the nucleosome. It is difficult to imagine such a way of transcribing nucleosomal DNA as a continuous run,

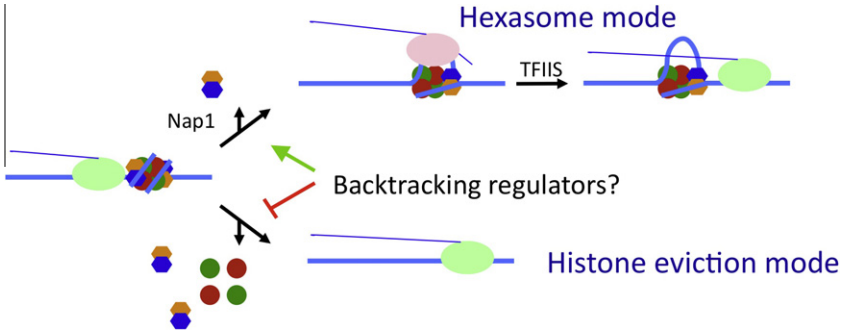

Fig. 4. Different modes of transcription elongation through chromatin may explain gene-specificity in RNA polymerase II backtracking. Transcription of chromatin without a complete nucleosome disassembly would favour RNA polymerase II backtracking, involving higher dependency on TFIIS and other arrest-solving factors. Full histone eviction during transcription elongation would prevent RNA polymerase backtracking. The first chromatin transcription mode would require H2A-H2B handling factors like Nap1, necessary to remove a single H2A-H2B dimer and to form the remodelled hexameric nucleosome (hexasome) that is competent for elongation. The eviction-dominated mode would require H3-H4 chaperons. Regulatory factors would control RNA polymerase II backtracking by modulating the predominance of these two alternative elongation modes in any gene.

but instead as a discontinuous phenomenon involving alternative transcription elongation and histones-DNA reconfiguration steps around the nucleosome. In this context, frequent RNA polymerase II backtracking would be expected (Fig. 4). A comparison made of the massive sequencing of nascent RNA with high resolution nucleosome positioning showed a paused distribution of elongating RNA polymerase II within nucleosomes [60], which is consistent with this hypothesis. In contrast, a transcriptional elongation mode dominated by histone eviction and complete nucleosome disassembly might prove more similar to in vitro naked DNA transcription and, therefore, might be less prone to backtracking, and less TFIIS-dependent (Fig. 4).

Gene-specificity in backtracking might arise from these alternative modes of transcription elongation. In some cases, as in the RP regulon, the hexasome-mediated, TFIIS-dependent elongation of nucleosomal DNA would be dominant. In other cases, such as SAGA-dependent inducible genes, complete histone eviction would be dominant and transcription elongation would depend much less on TFIIS and be less prone to backtracking. The observation of positioned nucleosomal profiles being more resistant to transcription in RP genes than in SAGA-dependent genes supports this model [51].

The existence of two alternative modes of transcription elongation, depending on the chromatin context, can help explain the viability of yeast mutants lacking TFIIS. In them, the histone eviction mode would rescue RNA polymerase II from general arrest. In this context, those regulatory mechanisms promoting the hexasome-mediated mode would enhance the dependence of RNA polymerase II on TFIIS and other reactivating factors. Factors like yeast Sfp1 fit in this regulatory role (Fig. 4).

5. Remarks

RNA polymerase II pausing during elongation is a common phenomenon. The connection between pausing and backtracking demonstrated in vitro and the involvement of TFIIS in the regulation of some genes during elongation suggest that RNA polymerase II backtracking is likely an important element in eukaryotic gene control. The conserved motives involved in stabilising backtracked RNA inside RNA polymerase II match this general backtracking role in gene regulation.

The comparisons made between the run-on and RNA polymerase II ChIP profiles in yeast are compatible with a significant proportion of backtracked RNA polymerases in certain gene families; e.g., RP genes. The transcriptional regulation of RP genes, in response to changes in the carbon source of the medium, involves changes in the proportion of backtracked RNA polymerases. This regulatory mechanism is mediated by transcription factor Rap1.

Backtracking requires either reactivation or RNA polymerase II removal mechanisms. Reactivation can be achieved by RNA cleavage, a process that is strongly stimulated by TFIIS. Reactivation can also be mediated by the Ccr4-Not complex in a process that does not involve RNA cleavage. For RP genes, TFIIS is required to resist the transcriptional stress imposed by NTP-depleting drugs like 6AU. This requirement depends on the regulatory programme imposed by specific transcription factors, like Sfp1.

What makes some genes particularly prone to RNA polymerase II backtracking remains unknown, but the emerging models proposed to explain chromatin transcription may help shed some light on this phenomenon. The precise characterisation of the mechanisms regulating RNA polymerase II backtracking is, therefore, one of the forthcoming challenges in the transcription research field.

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