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In vivo Studies of the Non-transcribed Spacer Region of rDNA in *Saccharomyces cerevisiae*

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Summary

The rDNA is the genetic locus that encodes the ribosomal RNAs and physically defines where ribosomes start to assemble. In the yeast *Saccharomyces cerevisiae*, its highly repetitive structure makes it a very interesting target for studies about genome stability, chromatin mediated transcriptional silencing and progression of aging. In fact, recombination among the repeated units is suppressed in a WT cell. Moreover, when genes transcribed by RNA polymerase II are inserted in the rDNA cluster, their transcription is silenced. Finally, the formation of rDNA minicircles has been shown to be one of the causes of aging in yeast. DNA topoisomerase I has been shown to suppress recombination specifically at the rDNA of *S. cerevisiae.* Moreover, also the chromatin structure of this locus is affected in a *top1* strain, because rDNA specific transcriptional silencing is abolished. Nonetheless, the molecular basis of how this enzyme interferes with these functions is yet unknown. Here are reported results obtained by *in vivo* studies of DNA-protein interactions occurring on the rDNA locus. The analyses include mapping of: nucleosome positioning; RNA polymerase I transcription factors and DNA topoisomerase I cleavage sites. Important conclusions can be drawn: nucleosome postitioning in the Non-transcribed Spacer (NTS) is not affected by RNA polymerase I transcription; the RNA polymerase I transcription factors bind DNA *in vivo* with a defined hierarchy, the DNA topoisomerase I cleaves the NTS in very specific sites, but cleavage is not induced by RNA polymerase I transcription. These *in vivo* studies help to characterize, the molecular basis of important phenomena as the transcriptional silencing and genome stability in yeast.

Key words: chromatin, DNA topoisomerase I, silencing, aging

Introduction

The ribosomal RNA genes (rRNA) in eukaryotes are arranged as tandemly repeated units and their transcription is specifically carried out by the RNA polymerase I *(1,2).* In the yeast *S. cerevisiae* the rDNA is present in one large tandem array (ranging from 150 to 200 units) *(3)* on the chromosome XII. In each single unit the 35S rDNA promoter consists of two elements: a core element and an upstream element *(4,5).* The Core Element (CE), essential for transcription, encompassing the transcriptional start site (about +8 bp) and extending to about –28/–38 bp from the RNA Initiation Site (RIS) *(4–6)*; the Upstream Promoter Element (UPE) stretching from position about –41/–51 bp to about –146/–155 bp and required for an efficient transcription *(4–6)*; moreover, an additional element connected with transcription is the Enhancer Element located at positions –2208 to –2396 bp

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from the RIS *(7–9)*, necessary for the stimulation of 35S RNA transcription when the cells are grown in a glucose-containing medium *(7)*. Nomura and colleagues, using a genetic approach *(10)*, identified the specific transcription factors required for RNA polymerase I transcription (the Core Factor, the Upstream Activating Factor and the protein called Rrn3). Further characterisation of the rDNA locus derives from the data obtained in the laboratory of Sogo *(11,12)* that suggested a transcriptional heterogeneity among the rDNA units; they showed that in a growing yeast cell only a randomly distributed fraction of the approximately 150 tandemly repeated rRNA genes is transcriptionally active. In addition, they determined the chromatin organisation by psoralen crosslinking, showing that the NTS is organised in nucleosomes, while the actively transcribing coding regions are free of these structures.

Chromatin organisation surrounding the RNA polymerase I promoter region has been studied by mapping the nucleosome positions. Furthermore, the DNA-protein interactions at the 35S promoter region have been investigated by *in vivo* footprinting. The interaction of the DNA topoisomerase I with the NTS has been also reported both by footprinting analysis and by induction of cleavage sites. This review describes in detail the DNA-protein interactions studied *in vivo* on the NTS region consisting of transcription factors, nucleosomes and DNA topoisomerase I. The relation with RNA polymerase I transcription is also discussed.

Chromatin Organization

The chromatin structure of NTS region shows different accessibility to psoralen in relation to the transcriptional activity of the flanking 35S gene *(11)*. Additional studies relative to the 35S coding region indicated that nuclesomes are present in the non-transcribed units and absent when transcription occurs *(12)*. The chromatin organization is mixed, with most of the NTS region organized in nucleosomes, the promoter being nucleosome free (preset-promoter) and the internal part of the transcriptional unit displaying only partly a nucleosomal structure *(13)*. A different approach, using micrococcal nuclease (MNase) digestion, showed that five well positioned nucleosomes occupy the area between the Pol I promoter region and the 5S gene and these nucleosomes are regularly spaced *(14)*. The region between the 5S gene and the enhancer is also arranged in nucleosomes, but these particles are much more delocalized. In agreement with a preset chromatin structure the promoter region with bound transcription factors is nucleosome free. The ARS region lies in a linker between nucleosomes region as expected for an origin of replication *(15,16)*.

The nucleosome positioning in the NTS is not sensitive to the transcription rate, in fact the positioning is maintained also in RNA polimerase I transcriptional mutants *(14)*. In addition to other functions *(17)*, also the DNA topoisomerase I has been reported to be involved not only in rDNA transcription *(18,19)* and recombination *(20)*, but also in its structural organization *(21)*.

In yeast, it was demonstrated that the activity of the ribosomal genes is heterogeneous, with only 50 % of the units actually transcribing *(12)*. On the other hand, the NTS has been observed to be always packaged in a nucleosomal array, but this chromatin structure differs in accessibility to psoralen in relation to the transcriptional activity of the flanking 35S unit *(11)*. MNase digestion does not show heterogeneity when cells are grown in the presence of glucose, either at low or high transcription rate. Actually, psoralen and micrococcal nuclease are very different tools which might reflect different stuctural characteristics and should therefore be interpreted in an integrated way.

Transcriptional Silencing

In yeast are present three mechanistically related forms of transcriptional silencing:

i) the repression of Mat α information at the HML locus, and of Mat α transcription at the HMR locus *(22, 23)*; *ii)* telomeric regions; *iii)* rDNA locus.

Eukariotic cells present a number of cases in wich the trascription state of a gene is affected by its position within the genome. The yeast *S. cerevisiae* shows a form of position-effect regulation, commonly referred to as transcriptional silencing *(24)* when genes are placed near telomeres or into rDNA locus, which is a form of transcriptional repression *(25–27)*.

In each case, *cis*-acting silencer elements are responsible for the repression of transcription in adjacent sequences and these elements interact with several trans-acting modifiers of position effect *(28,29)*. The essential role of histones in silencing *(30)* has led to the hypothesis that a heterochromatin-like comformation in these regions is responsible for transcriptional silencing.

The RDN1 locus has characteristics of heterochromatin. There are some evidences to support this conclusion:

i) the RDN1 locus consists of tandemly repeated DNA sequences, a situation analogous to that sequences in heterochromatic domain of higher eukaryotes *(31)*;

ii) in the RDN1 locus recombination is repressed, a characteristic similar to silenced regions of the *Drosophila* and *S. pombe* genomes *(15,20)*;

iii) the rDNA gene sequence contains *cis*-elements that are implicated in transcriptional silencing at telomeres and the HM loci, such as an autonomously replicating sequence (ARS; *29*), and Rap1p binding site.

It was recently demonstrated that transcriptional silencing affects Ty1 retrotransposons that integrate in various positions in RDN1 locus, resulting in repression of their transcription and, therefore, transposition *(26, 27)*.

Several trans-acting factors have been shown to characterize this phenomena, *i.e.* UBC2, SIR2, HTA1-HTB1 and TOP1. These factors are able to silence transcription of Ty1 element in RDN1 locus, but do not affect significantly Ty1 element functions outside the RDN1 locus, supporting the idea that silencing results from a specialized chromatin structure at RDN1 locus.

However, integration of the LEU2, URA3, and ADE2 genes in single copy into rDNA leads to their expression *(32,33)*, indicating that at least some Pol II trasnscribed genes can be expressed within rDNA *(27)*. Moreover,

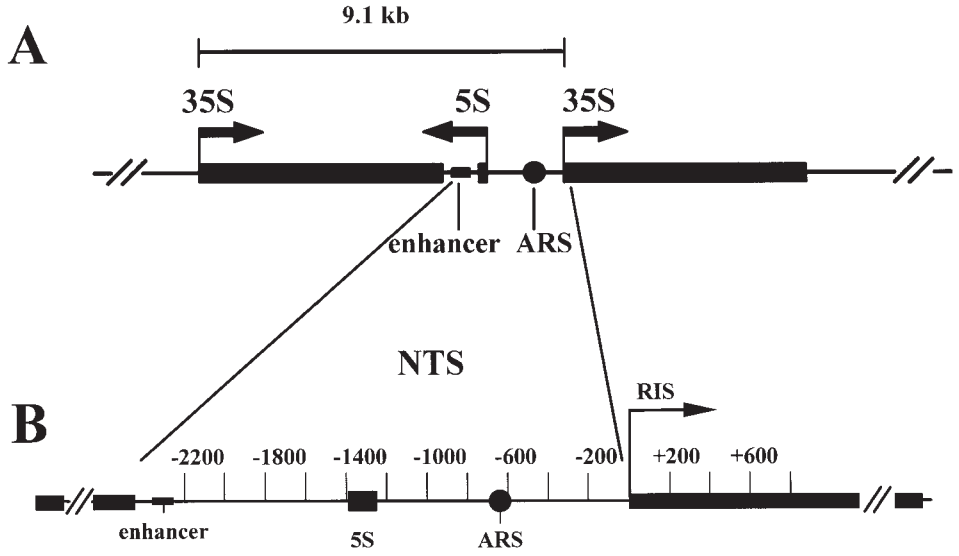

Fig. 1. Schematic representation of rDNA organization in *Saccharomyces cerevisiae*. The rDNA coding sequences; the arrows indicate 35S and 5S rRNA transcriptional initiation sites. **A)** enhancer and ARS elements are also indicated. The bar in the upper part of the figure indicates the dimension and the boundaries of the repeating unit. **B)** Enlargement of the Non-transcribed Spacer region. Numbering is relative to the 35S transcriptional start. The positions of most relevant elements (enhancer, ARS) are reported

rDNA silencing, like silencing at telomeres, depends on the strength of the particular promoter tested *(27)*.

RNA Polymerase I Transcription Factors

The RNA polymerase I specific transcription factors of *S. cerevisiae* have been isolated by a genetic screening. Nomura and colleagues took advantage of the fact that the 35S rRNA can be transcribed by RNA polymerase II when fused to the GAL7 promoter (*10*). This promoter is induced when cells are grown on galactose, but is repressed when glucose is used as a carbon source. Mutants defective in RNA polymerase I transcription will therefore be able to grow on galactose, but will be defective when grown on glucose. After random mutagenesis and selection for inability to grow on glucose containing media, 11 complementation groups, named rrn1-11, have been isolated (*10*).

Three of these *RRN* genes (*RRN1*, *RRN2* and *RRN4*) were shown to encode RNA polymerase I specific subunits (A190, A135 and A12.2, respectively) (*10*). Except for *RRN8*, which still has not been further characterized, all other *RRN* genes turned out to be transcription factors specific for RNA polymerase I (*10,34*).

The Upstream Activating Factor (UAF)

RRN5, *RRN9* and *RRN10* where not absolutely required for growth on glucose media, but resulted in extremely small colonies, reflecting a very reduced transcription of rDNA by RNA polymerase I. These gene products must therefore present a highly stimulating activity for transcription by RNA polymerase I. The *RRN5* gene encodes a 58 kDa hydrophilic protein with a highly acidic region near the carboxyl terminus of the molecule. Also *RRN9* encodes a highly hydrophilic protein of very similar size (50 kDa), while the protein encoded by *RRN10* is much smaller (17 kDa). These three proteins copurify together with a 30 kDa protein and the two core histones H3 and H4, but not with the other two histones H2A and H2B *(35,6)*. Whether H3 and H4 are actually part of this complex or whether this copurification reflects simply an interaction between this complex and the core histones is still an open question. This complex is necessary to form a stable preinitiation complex *in vitro*. When extracts of mutants with deletion of either *RRN5*, *RRN9* or *RRN10* genes were used for *in vitro* transcription, the RNA polymerase I was still able to transcribe a second template after preincubation with a first one. Adding the purified complex to the extract complemented the lack of formation of the preinitiation complex. This complementation was abolished when the first DNA template presented a deletion between position –155 to –119 respective to the RIS, indicating that these sequences represent the functional correlated *cis*-element. For the ability to stimulate RNA polymerase I transcription and for its functional interaction with the upstream promoter element, this complex has been named Upstream Activating Factor (UAF) (*35*).

The Core Factor (CF)

A second complex, the Core Factor (CF), consists of the association of Rrn6p, Rrn7p and Rrn11p and is essential for transcription by RNA polymerase I. Rrn6p is a large 115 kDa protein with a leucine zipper like motive near the amino terminus, while Rrn7p is a 58 kDa protein with a glutamine rich region. The biological significance of these motives is still unclear, because their homology to known motives of the same kind is not very striking (*36,37*). This complex is essential for basal transcription by RNA polymerase I, but is unable to form a stable preinitiation complex in the absence of UAF and a third factor, which is the TATA Binding Protein (TBP) (*38*).

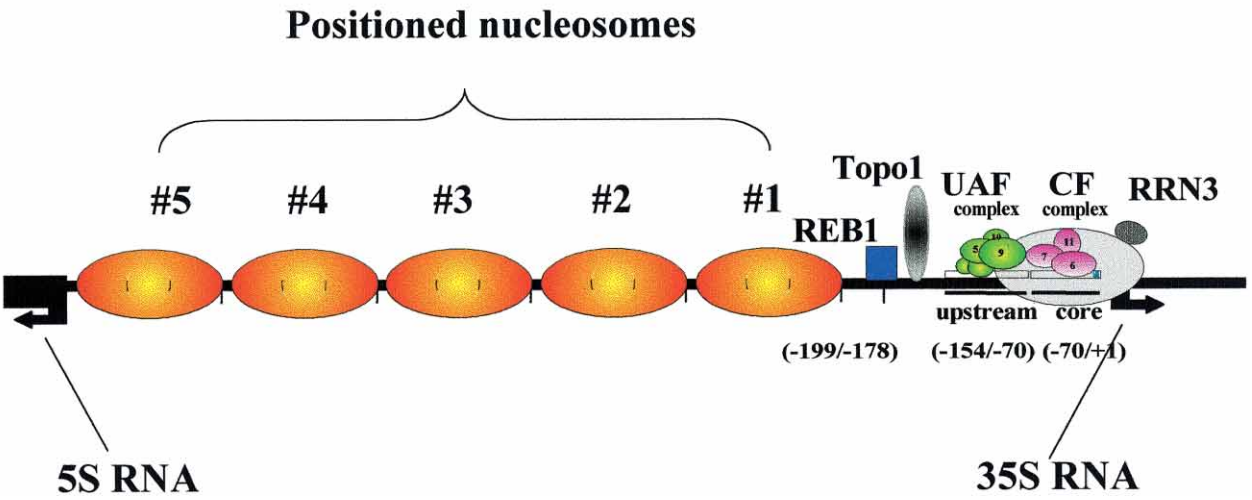

Fig. 2. Summary of the main DNA protein interactions occurring on the 35S RNA promoter and the upstream sequence. The numbering refers to the RNA initiation site

The Rrn3p

The *RRN3* gene encodes a 72 kDa protein containing a highly acidic region in the middle of the molecule, and is also essential for transcription by RNA polymerase I. This protein is not a part of the stable preinitiation complex, because when two preincubations, differing in the presence/absence of Rrn3p and DNA template are mixed prior adding nucleotides, both templates are transcribed equally. This protein is thought to directly interact with a component of the RNA polymerase I itself, helping to recruit the enzyme to the other factors each round of transcription (*39*).

Based on these data, Nomura and colleagues proposed a model for RNA polymerase I transcription, in which UAF would recognize and bind to the upstream promoter element. This would enable CF, together with the TATA Binding Protein, to contact the core promoter element, forming a »committed complex«. RNA polymerase I would associate initially and independently from DNA with Rrn3p. This interaction would finally permit the binding of RNA polymerase I/Rrn3p to the »committed complex«, forming the »initiation complex« and allowing transcription (Fig. 2, *38*).

Analysis of Mutants

The Core Factor (CF) and the Upstream Activating Factor (UAF) have been shown *in vitro* to bind the Core Element and the Upstream Promoter Element, respectively. By using *in vivo* footprinting techniques, we have analyzed DNA-protein interactions occurring at the rDNA promoter. We performed these experiments by digesting with DNase I nuclei from yeast strains differing in RNA polymerase I transcription efficiency. In particular, we have studied the following mutant strains : NOY 558, lacking the Rrn7p subunit of CF complex (*36*); NOY 699, lacking the Rrn5p subunit of the UAF complex (*35*); D1281-d, lacking the A43 subunit of the RNA polymerase I (*41*) and NOY 604, lacking Rrn3p, that is essential for transcription by RNA polymerase I. These mutants are viable in galactose medium, due to the presence of an episomal copy of the 35S rRNA under GAL7 promoter (plasmid pNOY 102 or pNOY103, (*10*)) transcribed by RNA polymerase II. In order to assign each footprint to each corresponding factor *in vivo*, we examined the DNase I sensitivity of the promoter region in mutants lacking CF or UAF and in WT condition (NOY 505). The digestion profiles were compared with deproteinized DNA digested *in vitro* with increasing amounts of DNase I, which reveals the intrinsic sensitivity of naked DNA to the nuclease. In the *rrn7* mutant, when compared to the WT, the loss of footprint in the putative binding site for CF complex was observed, while the region of the putative binding site for UAF was still protected from DNase I digestion. The *rrn5* mutant showed a loss of footprint in both the putative binding sites. We concluded that *in vivo* CF binds DNA in the region +1/–45 bp from the RNA Initiation Site and UAF binds DNA in the region –50/–160 bp from the RIS, in agreement with *in vitro* data. Moreover, since CF footprinting was lost when UAF was missing, we concluded that *in vivo* a binding hierarchy exists : the presence of UAF facilitates the binding of CF on rDNA promoter. To better understand the dynamics of rDNA transcription initiation, we extended the analysis to the strains mutated in *A34* and *RRN3*. When the two strains were treated *in vivo* with DNase I, the loss of CF footprint was again observed. These data suggest that a functional RNA polymerase I is necessary for the efficient binding of CF. Rrn3p has been reported to be one of the essential components of transcriptional apparatus working as an RNA polymerase I recruitment factor or as an elongation factor. Our data are consistent with the hypothesis of Rrn3p recruiting RNA polymerase I to the promoter and suggest that this interaction also stabilizes the binding of CF. Thus, *in vivo*, CF is recruited to the Core Element by UAF and stabilized on DNA by the interaction with RNA polymerase I and Rrn3p (Fig. 2)

DNA Topoisomerase I

The topological state of DNA inside a living cell is controlled by the combined action of a class of enzymes,

the DNA topoisomerases. They can be divided in two main classes for their ability to cut one (class I) or two (class II) DNA strains at a time (*17*).

DNA topoisomerase I is a class I enzyme that is essential in higher eukaryotes, while it is dispensable in yeast. This enzyme has been reported to be important for both replication and transcription, where it likely removes topological stress caused by the advancing DNA and RNA polymerases (*42–44*).

Beside these more general functions, DNA topoisomerase I plays a major role in the control of the metabolism of ribosomal DNA. In fact, in mammalian cells, the nucleolus is the cellular compartment where DNA topoisomerase I localizes preferentially, as shown by immunostaining (*45*). In this particular genomic locus it has been shown to interfere with transcription, chromatin mediated transcriptional silencing and mitotic recombination.

In higher eukaryotes, transcription of the 45S rRNA precursor by RNA polymerase I is inhibited by treatment with camptothecin, a specific inhibitor of DNA topoisomerase I (*46*). This drug stabilizes a reaction intermediate consisting of the enzyme covalently attached to the nicked DNA substrate (*47*). The inhibition occurs preferentially towards the 3' end of the transcript, probably due to a more severe topological stress in this region (*46*). The importance of DNA topoisomerases during transcription by RNA polymerase I has been confirmed by a study in *S. cerevisiae*. While initiation was not altered, elongation was severely inhibited in strains mutant for both DNA topoisomerase I and 2 (*18*). Genetic evidences provide a further link between DNA topoisomerase I and transcription by RNA polymerase I. The latter contains a subunit, A34.5, which has no homologues in the other two DNA dependent RNA polymerases. Cells lacking this subunit show only a very slight growth defect. The same deletion instead becomes lethal in a Δ*top1* background (*48*). Together, these studies suggest a direct involvement of DNA topoisomerase I in transcription by RNA polymerase I.

A second important function of DNA topoisomerase I specific to the rDNA locus is the suppression of mitotic recombination. In fact, the rDNA cluster represents a highly repetitive DNA sequence (*3*). Therefore a tight control mechanism must be in place to balance the propensity toward high levels of recombination in these directly repeated sequences, which potentially leads to loss of information (*49,50*). The frequency of mitotic recombination in the rDNA has been determined by measuring the loss of an inserted marker gene. This frequency increases 140–200 fold in the absence of DNA topoisomerase I (*20*).

The suppression of recombination in the rDNA locus is particularly intriguing, since a segment of rDNA actually stimulates recombination when placed outside the nucleolar environment (*51*). This segment, named HOT1, is identical to the sequences within the rDNA repeat that regulate transcription by RNA polymerase I, the 35S promoter and the enhancer (*52*). Moreover, the recognition activity is directly related to the transcription by RNA polymerase I. Any mutation inside this sequence, that compromises transcription, also reduces HOT1 activity (*52*). The mechanism by which HOT1 activity is suppressed in the nucleolus is still unknown. It will be interesting to investigate whether and how DNA topoisomerase I contributes to this suppression of recombination.

A study of DNA-protein interactions over the 35S promoter region by *in vivo* footprint revealed the presence of a protected region that became accessible to DNase I in the absence of DNA topoisomerase I (*14*). Furthermore, when spheroplasts are treated with camptothecin, a strong cleavage site can be observed at the same sequence, confirming the presence of DNA topoisomerase I in this location (*53*) (see Fig. 2 for detail of mapping). Site specific cleavage in the rDNA locus had been previously shown to occur also in the ciliate *Tetrahymena termophila* (*54*). In *S. cerevisiae*, two other preferential cleavage sites have been reported to occur in the upstream located enhancer element (*53*). At all three sites, the enzyme preferentially nicked the same DNA strand. It is interesting to notice that these sequences are all confined to the DNA segments that define HOT1.

The site specific cleavage of DNA topoisomerase I is also located closely to a replication fork barrier (RFB), that overlaps the enhancer element (*55,56*). The arrest of the replication fork over this sequence depends on the gene product of *FOB1* (*57*). In the absence of Fob1p, both RFB and HOT1 functions are abolished (*58*), although HOT1 function is independent of the stalling replication fork (*59*). Inside the rDNA cluster, a deletion of *FOB1* reduces the rate of rDNA recombination (*59*). Moreover, the hyperrecombination in the rDNA cluster, observed in a Δ*sir2* strain, is suppressed by the concomitant deletion of *FOB1* (*60*). In this functional context, the DNA topoisomerase I plays likely a critical role. In fact, a Δ*sir2* strain and a Δ*top1* strain show significant similarities in their phenotypes specific to rDNA. Both mutant strains show increased mitotic recombination in the rDNA locus. Moreover, transcriptional silencing of the rDNA is also abolished in a Δ*top1* mutant (*26,27*). Nonetheless, the molecular bases of how these proteins function together to guarantee the genomic stability of the rDNA locus are still unknown. The combined control of chromatin structure by Sir2p and DNA topology by DNA topoisomerase I, though, seem to counteract the genomic instability caused by a stalling replication fork.

Concluding Remarks

Although investigated since at least three decades, the rDNA locus continues to be of great interest. Recently, in yeast two important aspects of rDNA metabolism were discovered: *i)* a transcriptional silencing concerning genes transcribed by RNA polymerase II inserted in the rDNA repeats *(27),* and *ii)* the appearance of rDNA organized as extrachromosomal ring has been reported to be related with life span. Other important aspects of this genetic system are the unidirectional barrier to replication fork movement, the stability of HOT1 elements inside the nucleolus and the maintenance of the number of repeats. The molecular basis of these phenomena are still unclear although the role of chromatin structure was frequently hypothesized. The identification of specific factors that directly interact with the rDNA and

their relation to each other in different genetic backgrounds will certainly improve our understanding of this important genetic system.

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Istraživanje in vivo netranskribiranih međuprostora rDNA u *Saccharomyces cerevisiae*

Sažetak

rDNA je genetski lokus koji kodira za ribosomske RNA i fizički određuje gdje započinje slaganje ribosoma. U kvascu *S. cerevisiae*, njezin veliki broj kopija čini je zanimljivom za studije stabilnosti genoma, transkripcijskog utišavanja s pomoću kromatina, te tijeka starenja. Zapravo rekombinacije između ponavljajućih jedinica suprimirane su u stanicama divljeg tipa. Štoviše, ako se geni koje transkribira RNA polimeraza II ubace u nakupinu rDNA, njihova je transkripcija utišana. Nadalje, vidi se da je stvaranje minikružnih rDNA jedan od uzroka starenja kvasca. DNA topoizomeraza I specifično suprimira rekombinacije rDNA u *S. cerevisiae*. Nadalje, kromatinska je struktura toga lokusa izmijenjena u soju *top1* jer je narušeno rDNA specifično transkripcijsko utišavanje. Do danas je nepoznata molekularna osnova djelovanja tog enzima na te funkcije. U radu su prikazani rezultati *in vivo* proučavanja interakcije protein-DNA u rDNA lokusu. Analize obuhvaćaju mapiranje: pozicije nukleosoma, transkripcijskih faktora RNA-polimeraze I i mjesta cijepanja DNA-topoizomerazom I. Odatle slijede važni zaključci: pozicioniranje nukleosoma u netranskribiranom dijelu (NTS) nije poremećeno transkripcijom s RNA-polimerazom I; faktori transkripcije RNA-polimeraze I vežu se na DNA *in vivo* po određenom redoslijedu; DNA-topoizomeraza I cijepa NTS-regije vrlo specifično, ali to cijepanje nije inducirano transkripcijom s RNA-polimerazom I. Navedena *in vivo* istraživanja pomažu karakterizaciji molekularne osnove važnih pojava kao što su utišavanje transkripcije i stabilnost genoma kvasca.