FOB1 affects DNA topoisomerase I *in vivo* cleavages in the enhancer region of the *Saccharomyces cerevisiae* ribosomal DNA locus

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ABSTRACT

In *Saccharomyces cerevisiae* the *FOB1* gene affects replication fork blocking activity at the replication fork block (RFB) sequences and promotes recombination events within the rDNA cluster. Using *in vivo* footprinting assays we mapped two *in vivo* Fob1p-binding sites, RFB1 and RFB3, located in the rDNA enhancer region and coincident with those previously reported to be *in vitro* binding sites. We previously provided evidences that DNA topoisomerase I is able to cleave two sites within this region. The results reported in this paper, indicate that the DNA topoisomerase I cleavage specific activity at the enhancer region is affected by the presence of Fob1p and independent of replication and transcription activities. We thus hypothesize that the binding to DNA of Fob1p itself may be the cause of the DNA topoisomerase I activity in the rDNA enhancer.

INTRODUCTION

The genes coding for ribosomal RNAs in *Saccharomyces cerevisiae* are organized as a 9.1 kb basic unit repeated in tandem about 100–150 times (1) coding for the 35S RNA (further processed in 5.8S, 16S and 25S RNA), and the 5S RNA (2). This genetic locus is transcribed by two different specialized RNA polymerases: RNA polymerase I and RNA polymerase III, transcribing the 35S RNA and the 5S RNA, respectively. This locus is fundamental for yeast life, providing the whole RNA content of the ribosomal particles.

The combination of both *in vitro* and *in vivo* approaches (3–5) has established that the DNA–protein interactions occurring in the Non-Transcribed Spacer 2 region (NTS2) (see Figure 1), particularly at the 35S RNA promoter, are represented by a complex interplay of transcription factors acting to stimulate RNA polymerase I activity and other proteins like the Reb1p, or the DNA topoisomerase I enzyme, whose role in that particular region has not been established yet. *In vivo*, a fine mapping was obtained for UAF, CF, Reb1p and Top1p (5). Important events such as the termination of RNA polymerase I transcription (6) and the block of the DNA replication fork (7) occur at the NTS1 sequence (reported in Figure 1) and in particular at the enhancer region (E). However, only very recently DNA–protein interactions, have been reported for this region. In particular, it has been demonstrated (8) that Sir2p, Net1p and Fob1p bind to this portion of regulatory sequences. It has also been shown that binding occurs at the promoter region although with different efficiency. The method utilized Chromatin Immunoprecipitation (ChrIP), while representing one of the best analysis available to reveal site-specific DNA–protein interactions, does not allow mapping at nucleotide level of resolution. Furthermore, it has been shown that Fob1p can bind, *in vitro*, to two out of three replication fork block (RFB) sequences (9) providing a model for its activity in the block of the replication fork. The importance of *FOB1* gene is due to the following relevant findings: *FOB1* is required for rDNA recombination and for its replication fork blocking activity (10) to prevent collision between DNA replication and rDNA transcription events. In addition, it is necessary for either contraction or expansion of ribosomal units (11); *FOB1* deletion expands life span in yeast, also reducing the production of extrachromosomal rDNA circles (ERCs) (12). Moreover, it is involved in the control of transcriptional silencing occurring at the enhancer region of rDNA (8).

In previous analyses (13), we demonstrated that DNA topoisomerase I specifically cleaves DNA at two sites in the enhancer region and in one site at the 35S RNA promoter. Those regions overlap with those reported to be associated with Sir2p, Net1p and Fob1p (8).

It has been shown that DNA topoisomerase I has an active role in both transcriptional silencing (14) and in DNA recombination of rDNA units (15,16). Strikingly, all the genes

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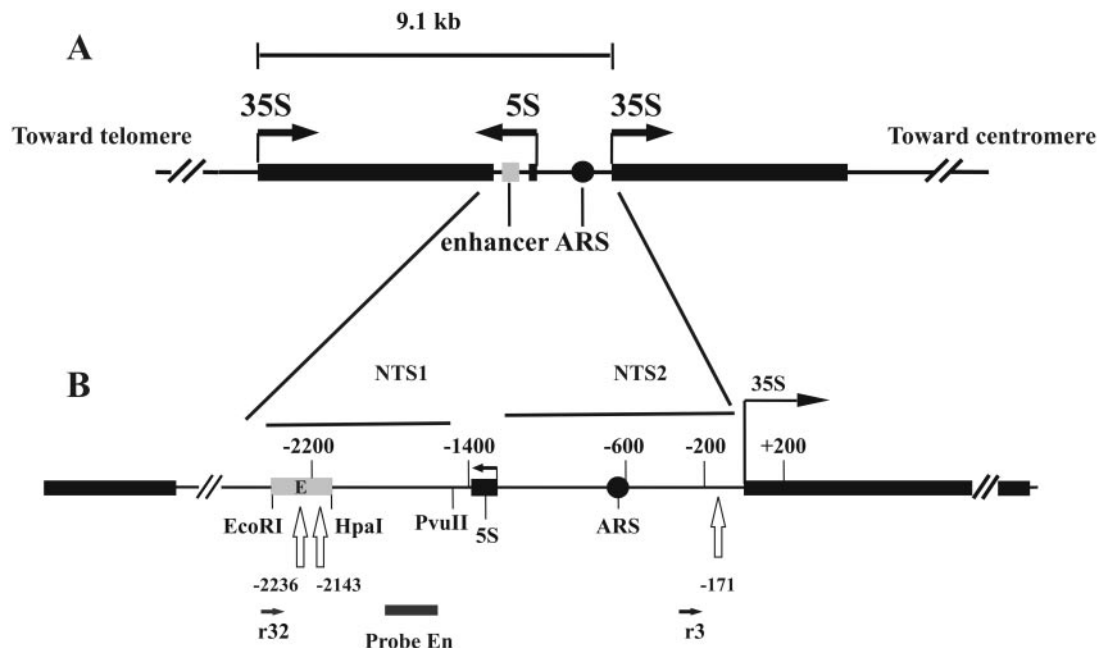
Figure 1. Schematic representation of rDNA organization in *Saccharomyces cerevisiae*. (**A**) The rDNA coding sequences: the black arrows indicate the 35S and 5S rRNA transcriptional initiation sites. The enhancer and ARS elements are also indicated. The bar in the upper part of the figure indicate the dimension and the boundaries of the repeated unit. (**B**) Enlargement of the NTS1 and NTS2. The positions of the most important DNA elements (Enhancer, E and ARS) and the main restriction enzymes are indicated. The positions of DNA topoisomerase I cleavage sites within the NTS1 and NTS2 regions are shown (empty arrows). The probe used for hybridization (Probe En) and the oligonucleotides used for the primer extension reactions are also reported (see text for details). Numbering is relative to the 35S RNA transcriptional start (+1).

involved in these pathways share similar phenotypes with DNA topoisomerase I. In particular, *TOP1*, *NET1*, *SIR2* and *FOB1* are all connected with ribosomal silencing. *SIR2* and *FOB1* are also engaged in DNA recombination of ribosomal units together with *TOP1*. In order to clarify the possible role, if any, of the DNA topoisomerase I in the ribosomal silencing or in rDNA recombination, we evaluated the *in vivo* binding of Fob1p to the rDNA locus by a high resolution method. In order to investigate the potential connection between Fob1p activity and the DNA topoisomerase I cleavage sites, we provide evidences that DNA topoisomerase I cleavage activity is dependent on *FOB1* in the NTS1, while *FOB1* independent is in the NTS2. We also show that events like DNA replication or rDNA transcription do not affect the DNA topoisomerase I site-specific reactions.

MATERIALS AND METHODS

Yeast strains, plasmids and culture media

The strains used in this study are: **W303-1a** (WT) (Mat**a**, ade 2-1, ura 3-1, his 3-11,15, trp1-1, leu 2-3112, can1-100); **D128-1D** (Δ43) (Mat**a**, rpa43::LEU2 ade 2-101 uaa, ura 3-52, lys2-801 uag, trp1-Δ63, his 3-Δ200, leu 2-Δ1/) pNOY102, kindly provided by P. Thuriaux; **Y1422** (sir2Δ): (Mat**a**, Leu2-3112 ura3-52 ade8, trp1 Δ901 sir2::TRP1), kindly provided by J.Broach; **NOY1064** (fob1Δ): (Mat**a**, ade 2-1, ura 3-1, his 3-11,15, trp1-1, leu 2-3112, can1-100, fob1::HIS3) and **NOY908** (rdnΔΔ pPolI) (Mat**a**, ade 2-1, ura 3-1, his 3-11, trp1-1, leu 2-3112, can1-100 rdnΔΔ::HIS3 carrying pNOY373) kindly provided by M. Nomura; **WY69**

(net1Δ): (Mat**a**, ade 2-1, ura 3-1, his 3-11,15, trp1-1, leu 2-3112, can1-100, net1::HIS5) kindly provided by D.Moazed. **RS1479** (tof2Δ) (Mat**a**, ade 2-1, ura 3-1, his 3-11,15, trp1-1, leu 2-3112, can1-100 tof2::URA3) kindly provided by R. Sternglanz.

Culture media. The culture media utilized for all cell growths were the complete YP (17). Addition of Glucose or Galactose at a concentration of 3% is indicated when appropriate.

Enzymes and chemicals

Restriction enzymes and Micrococcal nuclease (MNase) were purchased from Boehringer. DNAse I and T4 polynucleotide kinase were purchased from Roche; Vent(exo⁻) polymerase from New England Biolabs; Zymolyase 100T from Seikagaku, Tokyo, Japan; α-Mating factor from Sigma-Aldrich and radio-chemicals from Amersham.

Chromatin analysis

MNase treatments: Cells (100–200 ml grown to 0.5 OD) were pelleted and resuspended in 10 ml of a buffer containing 1 M sorbitol, 50 mM Tris–HCl (pH 7.5), 10 mM β-mercaptoethanol, in the presence of 0.05 mg/3×10^7 cells of Zymolyase 100T and incubated for 10 min at 30°C. The spheroplasts were harvested, resuspended in Nystatin buffer [50 mM NaCl, 1.5 mM CaCl$_2$, 20 mM Tris–HCl (pH 8.0), 1 M sorbitol and 100 µg/ml nystatin] (18) and divided into 0.25 ml aliquots. MNase (0.2, 0.4, 0.8 and 1.6 U) was added to each aliquot and the samples were incubated at 37°C for 15 min. The reaction was stopped with 1% SDS and 5 mM EDTA (final concentrations). Proteinase K (40 µg/sample) was added

and the samples kept at 56°C for 2 h. The DNA was then purified by three phenol/chloroform extractions and ethanol precipitation. RNase treatment was also performed.

Low resolution analysis

Indirect end-labelling analysis (19) was performed as follows: after treatment with the appropriate restriction enzymes, the samples were electrophoresed in 1.2% agarose gels (1.75 V/cm), transferred to BA-S 85 nitrocellulose membrane (Schleicher & Schuell), hybridized according to standard procedures and detected by autoradiography.

In vivo footprinting

Preparation of nuclei. Cells (150 ml grown to 0.4 OD_{600}/ml) were centrifuged and resupended in 10 ml of a buffer containing 1 M sorbitol, 50 mM Tris–HCl (pH 7.5) and 10 mM β-mercaptoethanol in the presence of 0.05 mg/3 × 10^7 cells of Zymolyase 100T and incubated for 10 min at 30°C. Spheroplasts were then washed once with 1 M Sorbitol and resuspended in Lysis buffer [Ficoll 18%, 20 mM K-phosphate buffer (pH 6.8), 250 µM EDTA, 250 µM EGTA, 1 µM Leupeptine, 1 mM phenylmethlysulfonyl fluoride (PMSF), 0.15 mM Spermine and 0.5 mM Spermidine]. Nuclei were prepared as reported with minor modifications (20).

DNAse I treatment. Nuclei were resuspended in digestion buffer [15 mM Tris–HCl (pH 8.0), 50 mM NaCl, 1.4 mM $CaCl_2$, 200 µM EDTA, 200 µM EGTA, 1 µM Leupeptine, 1 mM PMSF, 0.15 mM Spermine, 0.5 mM Spermidine and 5 mM β-mercaptoethanol] and divided into 0.2 ml aliquots. DNAse I (6 and 12 U) was added to each aliquot and the incubation was carried out for 5 min at 0°C. The reaction was stopped with 1% SDS, 5 mM EDTA (final concentrations). Proteinase K (40 µg/sample) was added and the samples kept at 56°C for 2 h. The DNA was then purified by three phenol/chloroform extractions and ethanol precipitation, followed by RNase A treatment.

Induction of Camptothecin (CPT) dependent DNA topoisomerase I cleavage sites

Spheroplasts were obtained as described above, resuspended in cleavage buffer [3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.0), 1 M sorbitol and nystatin 100 µg/ml] and aliquots were incubated with 0–100–200 µM CPT for 2 min at room temperature. The reaction was stopped with 1% SDS and 5 mM EDTA (final concentrations). Proteinase K (40 µg/sample) was added and the samples kept at 56°C for 2 h. The DNA was then purified by three phenol/chloroform extractions and ethanol precipitation, followed by RNase A. The samples where the addition of CPT was substituted by 0.8 M NaCl (final concentration) provide informations on DNA integrity.

Oligonucleotide primers and probes

The following synthetic oligonucleotides were used as primers in the extension reactions: r3: 5′-CGCGTTTCCGTATTTTC-CGC-3′, r32: 5′-GGGGCCTAGTTTAGAGAGAAGTAG-3′.

The r3 and r32 synthetic oligonucleotides used as primers in the extension reactions lie at positions −267/−248 bp (r3) and −2453/−2430 bp (r32), respectively, from the 35S RNA transcriptional initiation start (RIS) (sequence number +1;

see also Figure 1). According to standard procedures 5′ end-labelling using [γ-^{32}P]ATP and T4 polynucleotide kinase was performed (21). The labelled oligonucleotides were purified by PAGE. The probe En, annealing positions −1705/−1458 bp, from the RIS was labelled by random priming.

Multiple-round primer extension and detection of DNAse I footprintings and DNA topoisomerase I cleavage sites

Genomic DNA (0.1/0.2 µg) were reacted with 5 U of Vent polymerase and 100 000 c.p.m. of end-labelled oligonucleotide (specific activity 1–2 µCi/pmol). The samples were cycled five times through the following steps: 95°C for 5 min, 63°C or 70°C (for r32 and r3, respectively) for 10 min and 76°C for 3 min. The extension products were phenol extracted, ethanol precipitated, dissolved in formamide and dyes, and analysed in 6% denaturing polyacrylamide gel. The DNAse I footprints were detected by autoradiography.

RESULTS

In vivo Fob1p-binding sites are located in the surrounding region where DNA topoisomerase I cleaves the NTS1

In order to clarify whether the localization of the *SIR2*, *NET1* and *FOB1* gene products [reported to bind to the rDNA in the NTS1 and NTS2, (8)] may interfere with the site-specific activity of DNA topoisomerase I, described for the same regions (13), we evaluated the *in vivo* binding, at the nucleotide level, of the only protein capable to bind DNA: Fob1p (9). To accomplish this task we set up an *in vivo* footprinting assay by DNAse I, as described previously (22). WT and *fob1Δ* cells were grown in rich medium to the exponential phase (0.4 OD/ml); nuclei were prepared [as in ref. (1)] and aliquots were subjected to digestion with different amounts of DNAse I (Figure 2A). After the *in vivo* enzymatic treatment, DNA was extracted and purified. The digestion products were subjected to a primer extension with a [γ-^{32}P]ATP labelled oligonucleotide (r32, see map in Figure 1), annealing from the position −2453 to the position −2429, numbering from the transcriptional start site of 35S RNA (+1). The primer extension reactions were conducted in PCR conditions (see Materials and Methods). The linear amplification products were purified, separated by sequencing gel (23) and visualized by autoradiography. Mapping of footprinted positions was obtained comparing the digestion pattern of the samples with the molecular weights (M, in Figure 2) and the sequencing ladders (A, C in Figure 2). As reported in Figure 2, the comparison between the DNAse I digestion profiles of WT and *fob1Δ* samples, reveals interesting differences represented by enhanced (empty arrowheads) and protected (filled arrowheads) regions, strongly supporting that, *in vivo*, site-specific factor(s) are bound to rDNA. In fact at positions −2232 and −2220 two protections appear; at the 3′ of this protected region, (position −2200) a clear enhancement is also present. We identified the region encompassing these three sites as 'a' (see Figure 2C). Furthermore, three additional DNA sites show differences between the two
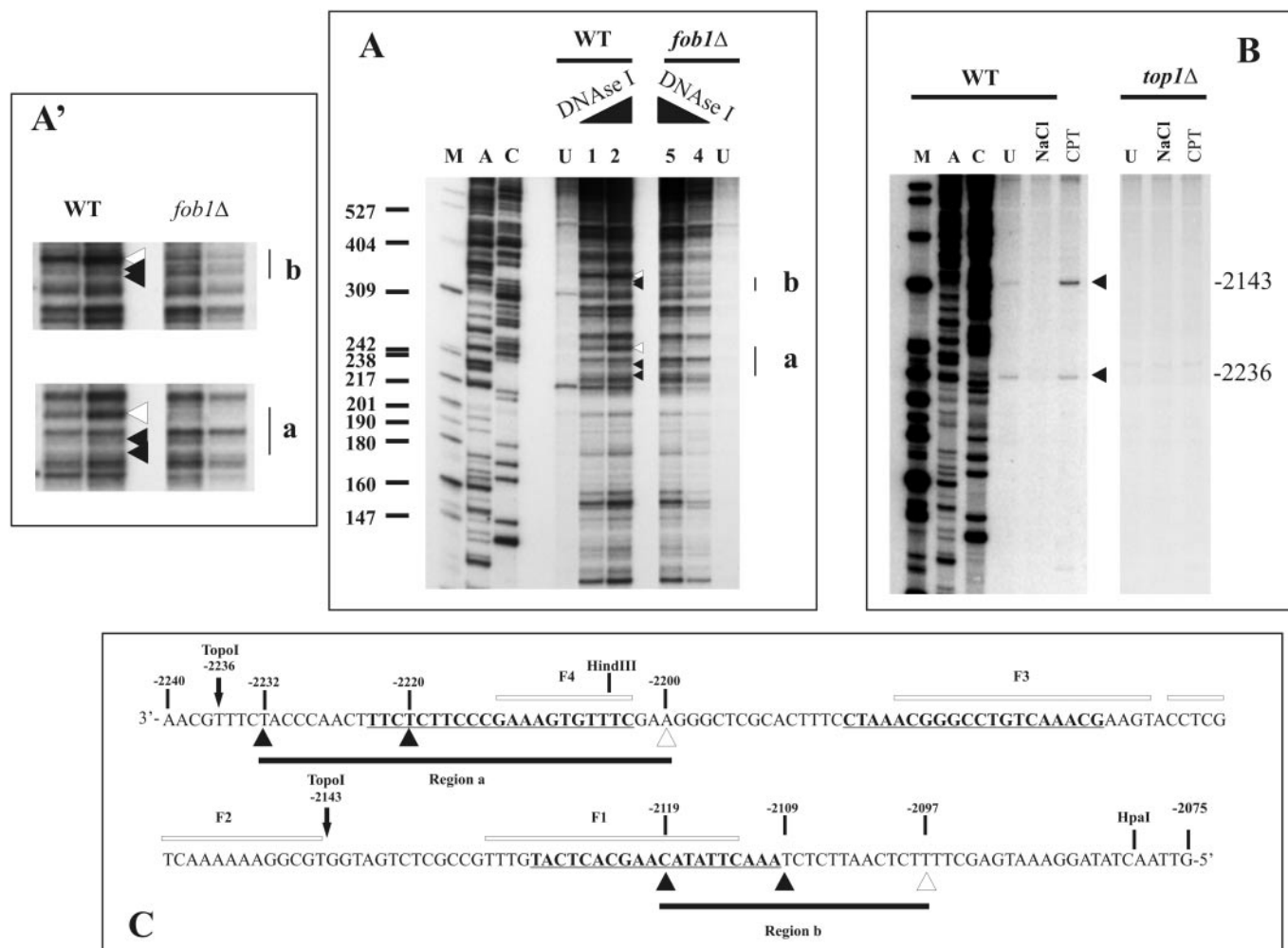
Figure 2. Detection of Fob1p footprinting and DNA topoisomerase I cleavage sites in the NTS1 region. (**A**) High resolution analysis of *in vivo* Fob1p footprinting. Comparison between WT and *FOB1* mutant. Starting from purified nuclei, chromatin was digested *in vivo* with 0–6–12 U of DNAse I for 5′ at 0°C (lanes U, 1 and 2 for WT and U, 4 and 5 for *fob1* mutant, respectively); after DNA purification the samples were primer extended with the labelled oligonucleotide r32 (see Materials and Methods and Figure 1 for map position and details). Lane M: size marker (pBR322/MspI); lane A, C: sequencing lanes; lane U: untreated samples. Protected areas are indicated by filled arrowheads while enhanced bands are shown by empty arrowheads; triangles indicate increasing amount of DNAse I in the treatment. Region 'a' and 'b' where Fob1p footprints DNA in the NTS1 region are also reported. (**A′**) Enlargement of the NTS1 region encompassing regions 'a' and 'b'. Enhancements and protection within the 'a' and 'b' regions are indicated by empty and filled arrowheads, respectively. (**B**) High resolution analysis of *in vivo* DNA topoisomerase I cleavage sites within the NTS1 region in WT strain and *top1*Δ strains. Lanes CPT and DNA from WT or *top1*Δ. Spheroplasts treated *in vivo* with 50 µM CPT; lane U: spheroplasts without treatmet of CPT; lane NaCl: spheroplasts pre-treated with 0.8 M NaCl. All samples were primer extended by Vent (exo⁻) DNA polymerase in PCR conditions, starting from 5′ labelled oligo r32 (for the position see Figure 1). M: size marker (pBR322/MspI); lane A and C: sequencing lanes. The canonic two previously mapped DNA topoisomerase I cleavage sites in the NTS1 region are indicated by filled arrowheads. Numbering refers to the 35S transcriptional start (+1). (**C**) Schematic representation of the overlapping *in vivo* and *in vitro* Fob1p-binding regions. This portion of the NTS1 sequence encompasses the three RFBs (underlined and bold). Strong (F1 and F4) and weak (F2 and F3) Fob1p-binding sequences previously mapped *in vitro* (15) are represented by empty thin rectangles. Regions 'a' and 'b' [corresponding to the 'a' and 'b' areas in (A) and (A′)], are here indicated by filled thin rectangles. Empty and filled arrowheads represent enhancements and protections respectively, mapped as reported in (A). Positions of the CPT dependent DNA topoisomerase I cleavage sites in the NTS1 [corresponding to those indicated by filled arrowheads in (B)] are also indicated. All numbering refers to the 35S transcriptional start and starts from RNA initiation site (+1).

digestion profiles: positions −2119 and −2109 are protected while a strong enhancement is present at position −2097. We named the region encompassing these three sites as 'b'. Conversely all the remaining DNA regions show the same DNAse I pattern in the two strains. In order to better evaluate the different DNAse I digestion profiles occurring in these regions ('a' and 'b') we enlarged the gel area reported in Figure 2A′. The protections/enhancements profile between regions 'a' and 'b' clearly describes the specific interaction(s) of putative protein(s) in WT cells, as compared to *fob1*Δ strain.

Since the two strains differ exclusively for the presence of the *FOB1* gene (shown in Figure 2) it is high likely that the regions 'a' and 'b' correspond to Fob1p-binding sites *in vivo*. In addition, comparing the data obtained *in vitro* with the HindIII–HpaI portion of the NTS1 sequence and the purified protein, a close match with our findings is noticeable (9). In fact, the 'a' region reported in Figure 2 overlaps with RFB3 (from −2232 to −2200 *in vivo* and from −2213 to −2203 *in vitro*) and the region reported as 'b' overlaps with RFB1 (from −2119 to −2097 *in vivo* and from −2129 to

−2113 *in vitro*. See also the scheme reported in the bottom of Figure 2C).

In order to show the strong overlapping between the *in vivo* Fob1p-binding sequences and DNA topoisomerase I specific cleavage sites in the NTS1 [observed previously, (13)], we treated WT cells with CPT, a specific DNA topoisomerase IB inhibitor (24). Spheroplasts obtained from WT or *top1Δ* cells (as reported previously) were permeabilized by nystatin (18) and treated with 0.8 M NaCl in order to remove most of the DNA topoisomerase molecules bound to DNA and to show the basal level of DNA integrity without the DNA topoisomerase I nicking activity. The DNA from treated cells was purified as reported (13) and subjected to primer extension in the same condition employed for the DNAse I treated samples. The purified material was loaded on a sequencing gel and the separated fragments were revealed by gel autoradiography. As reported in Figure 2B, the two major cleavage sites lie very close to the footprinted regions observed with the DNAse I analysis (compare Figure 2A and B) while in the top1D strain no cleavage production is observed.

The arrowhead labelled as −2143 in Figure 1 indicates the cleavage site located at the 5′ of the region 'b' (reported in the graphical representation of Figure 2C); this map position is quite different from that shown in a previous report (13): the site is actually the same but the attribution of map position was corrected by shifting 45 bp away from the 35S RNA transcriptional start, used as numbering reference. The other cleavage site, occurring at position −2236, at the 5′ of the region 'a' (Figure 2C) is also indicated by an arrowhead.

Previous experiments (8) have demonstrated, by ChrIP, that Sir2p, Net1p and Fob1p are bound to NTS1 and NTS2 sequences. In order to verify whether the Fob1p-binding on RFB sequences is maintained in *sir2Δ* or *net1Δ* strains we performed *in vivo* DNAse I footprinting in these mutants. We found that the two regions of Fob1p-binding ('a' and 'b') are still perturbed in the DNAse I digestions compared to the naked DNA profile (data not shown); therefore we can conclude that Fob1p-binding to the 'a' and 'b' regions is *SIR2* and *NET1* independent.

From the data reported so far, we can conclude that Fob1p-binding *in vivo* and *in vitro* (9) coincide, that the binding is *SIR2* and *NET1* independent, and that these specific-sites are nearly overlapping with the cleavage sites produced, *in vivo*, by DNA topoisomerase I.

### The DNA topoisomerase I cleavage sites of the enhancer region are FOB1 dependent

Given the strict colocalization of the Fob1p footprintings (Figure 2A) and the DNA topoisomerase I cleavage sites on the enhancer region of the rDNA locus (Figure 2B), and considering the colocalization of Sir2p, Net1p and Fob1p described previously (8), we wondered whether the DNA topoisomerase I cleavage sites are correlated with the presence of *SIR2*, *NET1* or *FOB1* functioning genes.

Thus, we studied the cleavage sites of DNA topoisomerase I in different strains lacking these genes. We assayed, by CPT blocking of DNA topoisomerase I activity, both the enhancer and the promoter regions in WT, *sir2Δ*, *fob1Δ* and *net1Δ* cells (Figure 3A and B, respectively). In the enhancer region, the characterization of the cleavage sites was obtained by primer

extension using the oligonucleotide r32 (see map in Figure 1). Samples reported as U correspond to untreated cells and show the cleavage efficiency of DNA topoisomerase I in the absence of CPT. Samples named NaCl indicate cells pre-treated with 0.8 M NaCl that prevents cleavage induction, and samples 1 to 8 represent couples of treatments with 100 and 200 μM CPT. The results shown in Figure 3A, clearly indicate that only the *fob1Δ* mutant completely lacks these cleavage sites, in any tested condition (0, NaCl, 100 e 200 μM CPT, respectively) when compared with the other three strains. Moreover, in WT, *sir2Δ* and *net1Δ* strains a very similar pattern shows, up with two DNA topoisomerase I cleavage sites (arrows in Figure 3A), suggesting that the presence/absence of Sir2p and Net1p does not basically interfere with the site-specific activity of DNA topoisomerase I in the rDNA enhancer region. We also studied the cleavage induction in the promoter region of the 35S rRNA, where colocalization of Sir2p, Fob1p and Net1p also occurs (8). The same DNA preparations used for the analysis reported in Figure 3A, were primer extended by oligonucleotide r3, annealing from position −268 to position −249 from the transcriptional start site of 35S RNA (Figure 1, for mapping details). The elongation products were separated by sequencing gel electrophoresis and the bands revealed by gel autoradiography. In the promoter region only one specific-site at −171 bp, due to the DNA topoisomerase I activity, is evidenced (black arrow in Figure 3B). While the *fob1Δ* strain in the enhancer region does not show any cleavage site, all the four strains show comparable cleavage pattern by DNA topoisomerase I in the promoter region (compare Figure 3A and B).

Taken together, these data indicate that the DNA topoisomerase I site-specific activity depends on *FOB1* in the rDNA enhancer region, but it is *FOB1* independent on the promoter region. The DNA topoisomerase I activity is also independent of the presence of Sir2p and Net1p both at the enhancer and at the 35S RNA promoter. This observation recalls recent findings (8) concerning the association of the regulator of nucleolar silencing and telophase exit (RENT) complex with the rDNA (25), where the authors described that both this association and the ribosomal silencing are *FOB1* dependent in the enhancer region (8), while they are independent in the promoter, suggesting the involvement of different factor(s). It is to notice that both the DNAse I and DNA topoisomerase I cleavage site assays, show two faint bands in most of the untreated samples (U in Figure 2A and Figure 3A), analysed by extension of the oligo r32. We believe that these bands have been originated by the treatment of the 'U' samples (as well as all the other samples) with the denaturing agent SDS that is used to stop the DNA topoisomerase I ongoing reactions (see Material and Methods for details). This procedure induces trapping of covalent complex of DNA topoisomerase I-DNA (26), thus revealing cleavages. This interpretation is supported by the absence of these faint bands in the *fob1Δ* untreated samples, observed in the DNAse I (Figure 2A, lane U *fob1Δ*), and in the DNA topoisomerase I experiments (Figure 3A, lane U *fob1Δ*), and in *top1Δ* (Figure 2B, lane U, *top1Δ*).

We further investigated two additional aspects of the DNA topoisomerase I site-specific cleavage reaction: (i) The reactivity of the enzyme on a substrate located outside the chromosome XII. In order to accomplish this we analysed a strain
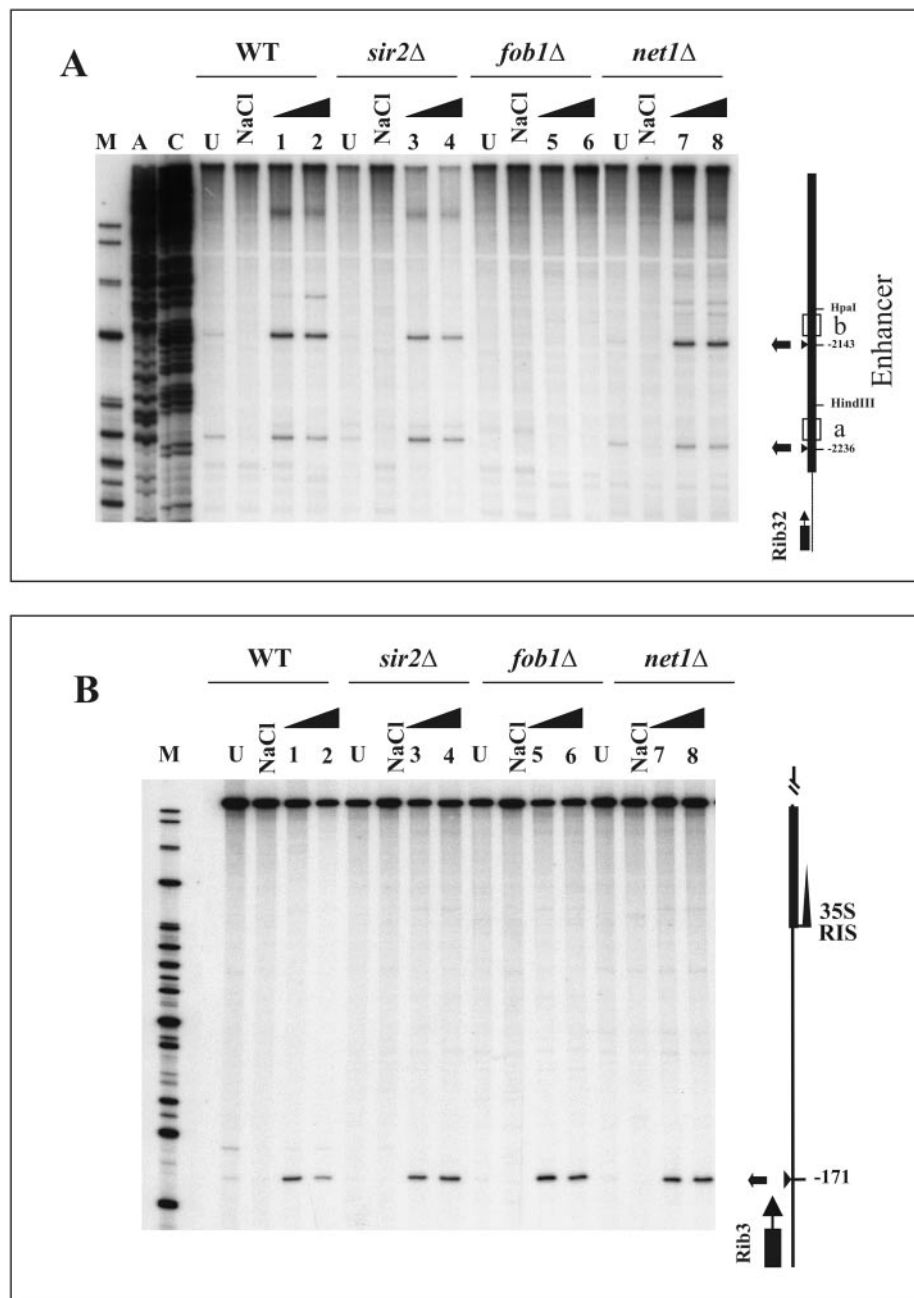
Figure 3. Site-specific *in vivo* cleavages by DNA topoisomerase I in the NTS1 and NTS2 regions. Comparison among WT, *sir2*, *fob1* and *net1* mutants. (**A**) High resolution analysis in the NTS1. Lanes 1 and 2, 3 and 4, 5 and 6, 7 and 8: spheroplasts from WT, *sir2*Δ, *fob1*Δ and *net1*Δ cells, respectively, treated *in vivo* with increasing amounts (filled triangles) of CPT (100 and 200 μM, respectively). Lanes U: control spheroplasts without CPT; lanes NaCl: spheroplasts pre-treated with 0.8 M NaCl. All samples were primer extended by Vent (exo⁻) DNA polymerase in PCR conditions, starting from 5′ labelled oligo r32 (for the position see Figure 1B). M: size marker (pBR322/MspI); lane A and C: sequencing lanes. The schematic (not in scale) map on the right part of the Panel represents the investigated region: positions of the two DNA topoisomerase I cleavage sites (−2236 and −2143) are indicated by filled arrows. Numbering refers to the 35S transcriptional start (+1). Regions 'a' and 'b' are also represented as empty rectangles. (**B**) High resolution analysis in the NTS2. Lanes 1 and 2, 3 and 4, 5 and 6, 7 and 8: spheroplasts from WT, *sir2*Δ, *fob1*Δ, *net1*Δ cells, respectively, treated *in vivo* with increasing amounts (filled triangles) of CPT (100 and 200 μM, respectively). Lanes U: control spheroplasts without CPT; lanes NaCl: spheroplasts pre-treated with 0.8 M NaCl. All samples were primer extended by Vent (exo⁻) DNA polymerase in PCR conditions, starting from 5′ labelled oligo r3 (for the map position see Figure 1). M: size marker (pBR322/MspI). The schematic (not in scale) map on the right part of the Panel represents the investigated region: the position of the unique DNA topoisomerase I cleavage site (−171) produced by the enzyme in the NTS2 region is indicated by the filled arrow. Numbering refers to the 35S transcriptional start (+1).

that does not contain the rDNA repeats [rdnΔΔ, (27)], and whose growth is supported by a 2 micron based plasmid carrying an entire ribosomal unit including the NTS sequence, pNOY373 (27). When cleavage sites are induced by CPT as reported above, no difference were observed with respect to the WT condition, suggesting that it is not the chromosomal context that induces the DNA topoisomerase I cleavage sites (data not shown), (ii) we asked whether the complex

Net1p-Sir2p, in association with Tof2p [all recently shown to be present in the RENT complex, (28)] could affect the DNA topoisomerase I cleavage. We performed the DNA topoisomerase I cleavage assay in a *tof2Δ* strain: no differences were observed relative to the WT strain (data not shown), thus confirming that the RENT complex is not involved in DNA topoisomerase I cleavage site production.

We set up a series of experiments involving the DNA replication block, the rRNA transcription suppression as well as a chromatin structure analysis, to understand whether the DNA topoisomerase I cleavage sites on the rDNA enhancer are to be attributed to (i) the blocking of the replication fork over the RFB sequences, (ii) the swivel activity necessary to release the torsional stress created by the RNA polymerase I transcription or (iii) an alteration of the chromatin structure.

DNA topoisomerase I activity in the rDNA enhancer is unaffected when DNA replication is blocked or rDNA transcription abolished

We evaluated the possibility that the RFB, exerted by Fob1p on RFB sequences, may be the physical cause that stimulates the site-specific activity of DNA topoisomerase I on the −2143 and −2236 sites. In fact, the position of these sites matches quite well with the RFB sequences (see the schematic drawing in Figure 2C). We analysed the DNA topoisomerase I cleavage sites after addition of 10 µg/ml of α-factor to WT cells grown to exponential phase (0.4 OD/ml), to block cell cycle progression and DNA replication (29). After a 2.5 h treatment the cells were checked for bud disappearance and processed for DNA topoisomerase I cleavage sites induction as

depicted in Figure 2 and 3. The results are reported in Figure 4A. The two cleavage sites, at position −2143 and −2236, are present in both untreated and α-factor treated cells suggesting that stalled forks (occurring only in the untreated cells) are not responsible for the induction of DNA topoisomerase I activity.

We also investigated the possibility that the collision between the blocked forks and the transcription bubble progressing from the adjacent unit, in the rDNA cluster, could represent a triggering event for cleavage. We studied the yeast strain Δ43, lacking the A43 subunit of RNA polymerase I (30), that is unable to support rDNA transcription. The viability of this strain in galactose medium is allowed in the presence of an episomal plasmid carrying a copy of the 35S RNA under the GAL7 promoter [plasmid pNOY102, (31)]. When we induced the DNA topoisomerase I cleavages sites, the comparison between the WT and the Δ43 strains did not show any difference in the site production (Figure 4B). Both strains show a similar pattern, suggesting that cleavage induction is not due to the collision between blocked forks and the transcription bubble.

The chromatin organization of both enhancer and promoter regions does not change in the fob1Δ strain

An alternative explanation for the loss of DNA topoisomerase I cleavage sites within the enhancer region in the *fob1Δ* strain could be compatible with an altered nucleosome organization in the strain lacking the *FOB1* gene. Previously, we studied the NTS2 region of rDNA, where we found five well positioned nucleosomes (5). In the same study we also investigated the
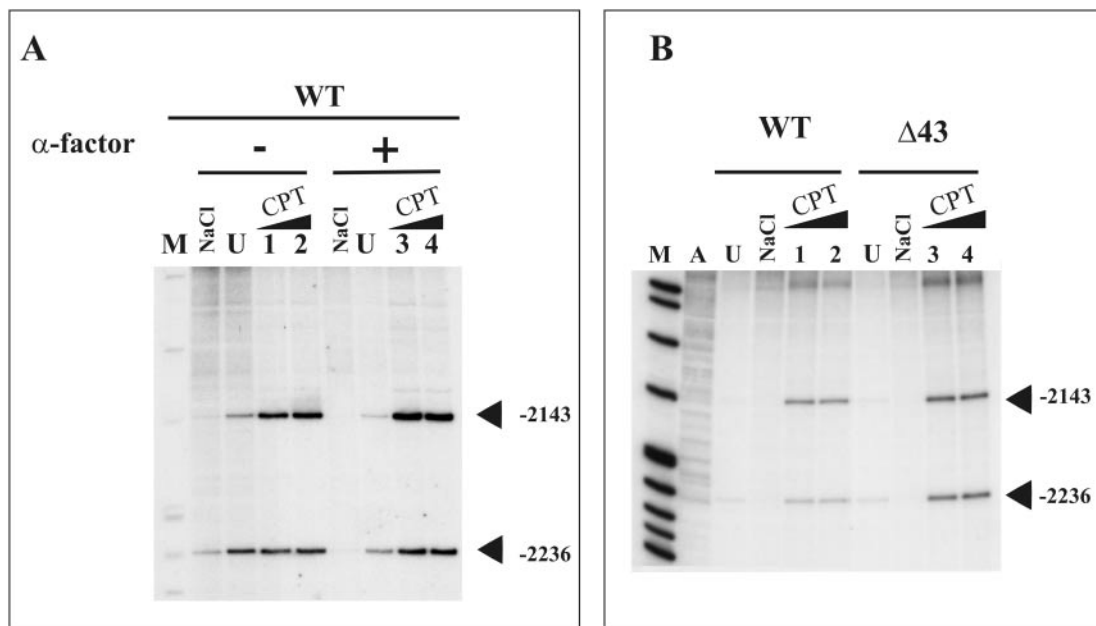
Figure 4. DNA topoisomerase I cleavage sites dependence on replication and transcription activities. (**A**) Replication dependence. WT cells from an exponential colture were synchronized in to S-phase by an α-factor arrest (+). WT cells from an exponential colture un-induced with α-factor were used (−) as control. Lanes 1 and 2: spheroplasts treated *in vivo* with increasing amount of CPT (filled triangles) as reported for Figure 3, in absence of α-factor; lanes 3 and 4: spheroplasts from synchronized cells treated *in vivo* with increasing amounts of CPT; lanes U (−/+): control spheroplasts without CPT; lanes NaCl (−/+): spheroplasts pre-treated with 0.8 M NaCl. All samples were primer extended by Vent (exo⁻) DNA polymerase in PCR conditions, starting from 5′ labelled oligo r32 (for the position see Figure 1B). M: size marker (pBR322/MspI); lane A and C: sequencing lanes. Positions of the two DNA topoisomerase I cleavage sites (−2236 and −2143) are indicated by filled arrowheads. Numbering refers to the 35S transcriptional start site (+1). (**B**) Transcription dependence. Comparison between WT and Δ*43* mutant. WT and Δ43 mutant spheroplasts from exponential coltures were treated with increasing amounts of CPT (filled triangles), as reported for Figure 3.

entire NTS region by nucleosome spacing analysis and concluded that a regular nucleosome structure is present in the enhancer region. In order to show whether the NTS1 chromatin could be altered in *fob1Δ*, we determined nucleosome positioning by end-labelling analysis (19) comparing the *fob1Δ* strain with the WT strain.

Cells from exponentially growing conditions (0.4 OD/ml) were treated as reported in ref. (5). Spheroplasts from cells grown in complete YPD medium, treated with nystatin to allow permeabilization, were digested with different amounts of MNase and DNA was purified. In order to evaluate whether the nucleosomes were regularly positioned, a specific restriction with PvuII of the MNase treated samples was coupled to hybridization with the En probe (mapping in Figure 1). The DNA fragments were then separated on 1.2% agarose gel and transferred onto a nitrocellulose filter by Southern blotting. The results are reported in Figure 5A. The *in vivo* treated samples (Chr) were compared with deproteinized DNA (which provides the sensitivity of the naked DNA toward MNase), both of them previously digested with PvuII. This comparison does not show defined protections thus confirming that nucleosomes in the enhancer region are not organized as precisely positioned array. This result would also be compatible with nucleosome absence, but their presence in this region was demonstrated in a previous work (5).

When the same analysis reported above was employed using the *fob1Δ* mutant, no differences were observed relative to the WT profile, [compare Figure 5B (*fob1Δ*) with A (WT)]. Also in this experiment no protection from MNase digestion is visible compared to the naked DNA analysis, so that we can conclude that nucleosomes in both WT and *fob1Δ* mutant in the enhancer region are not positioned.

The results reported in Figure 4 and 5 exclude that the *FOB1* dependence of the DNA topoisomerase I cleavage sites is due to the RFB activity, or to an alteration in the chromatin organization. Considering the close vicinity of the footprinted regions and the DNA topoisomerase I cleavage sites (shown in Figure 2A and B), we conclude that the Fob1p-binding itself allows DNA topoisomerase I to specifically cleave DNA at positions −2143 and −2236.

DISCUSSION

Fob1p acts binding in the enhancer region of the *S.cerevisiae* rDNA, where it controls the recombination of the ribosomal
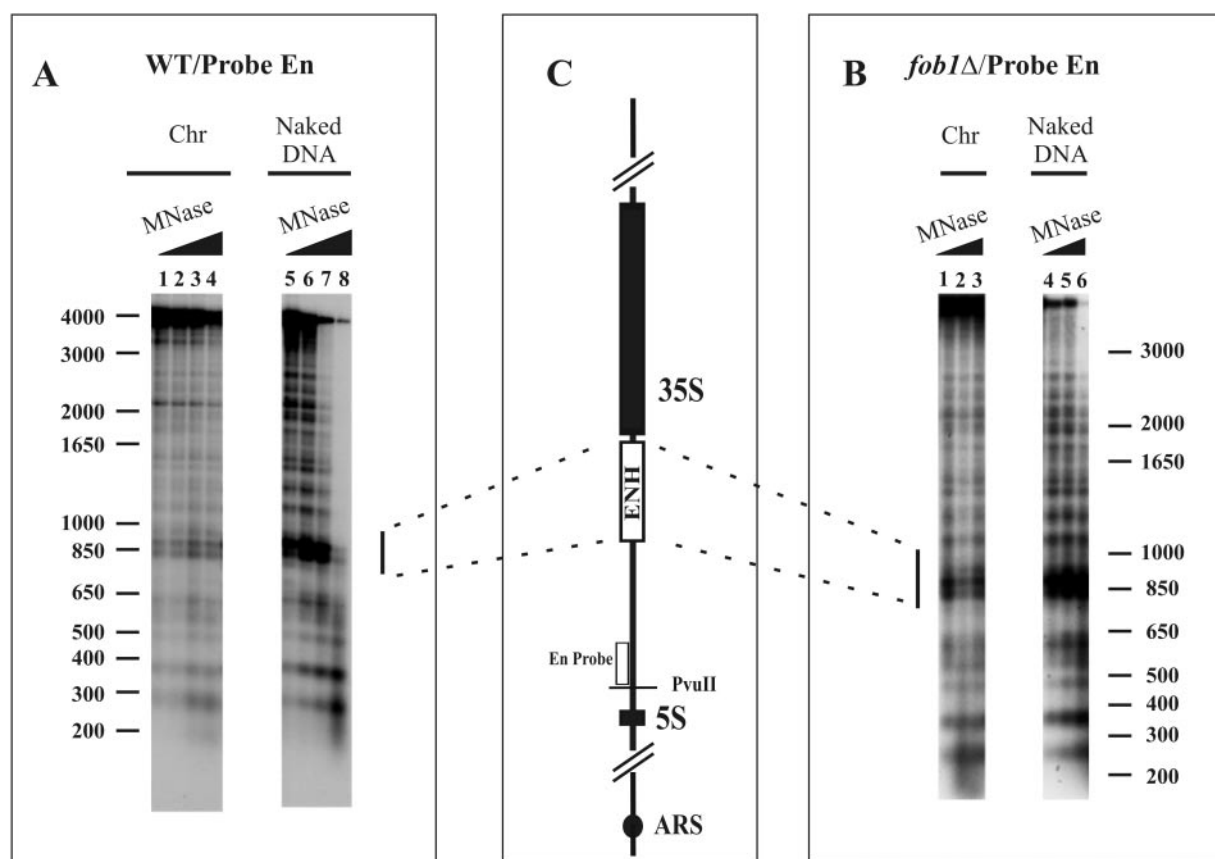
Figure 5. Chromatin organization of the NTS1 region in WT and fob1 mutant strains. (**A**) Nystatin permeabilized WT spheroplasts (see Materials and Methods) were treated with 0.2, 0.4, 0.8 and 1.6 U of MNase (triangles). The purified DNA was restricted with PvuII (see Figure 1), transferred onto a nitrocellulose membrane and hybridized with the En probe (see Figure 1B and Materials and Methods for position). Lanes 1–4: *in vivo* digested DNA (Chr). Lanes 5–8: *in vitro* digested samples of deproteinized DNA (Naked DNA). (**B**) *fob1* mutant strain was grown as for the WT and the obtained spheroplasts were treated *in vivo* with 0.2, 0.4 and 0.8 U of MNase (triangles). After digestion with PvuII restriction enzyme, the purified DNA was treated as reported for WT and hybridized with the En probe. Lanes 1–3: *in vivo* digested DNA (Chr). Lanes 4–6: *in vitro* digested samples of deproteinized DNA (Naked DNA). Bars and numbers at the left part of each gel, refer to molecular weight markers positions (1 kb plus ladder). (**C**) Schematic representation of the enhancer and its surrounding region.

units (10). We mapped, at nucleotidic resolution level, the *in vivo* binding sites of this protein, within the NTS1 region of the rDNA. In fact, it has recently been demonstrated *in vitro,* that this protein is capable to bind, with different efficiency, the three RFB sequences (RFB1-3) lying in the NTS1 tract (9). Moreover, it has been reported that Fob1p together with Sir2p and Net1p localizes *in vivo* both in the NTS1 (enhancer) and in the NTS2 (35S RNA promoter) although with different distribution (8). However the *in vivo* approach utilized (chromatin immunoprecipitation) did not provide information with the nucleotide level resolution due to the method approximation ranging around 100–200 bp.

Using an *in vivo* footprinting assay we mapped the binding sites of the *FOB1* gene product, (reported in Figure 2) showing a good correlation with those mapped *in vitro* and reported previuosly (8). As for the *in vivo* analysis, the best interactions are between Fob1p and the RFB1 and 3, respectively, being the RBF2 sequence not bound *in vivo* and weakly bound *in vitro* (compare the graphical scheme in Figure 2C). The most important output of this fine mapping obtained *in vivo*, is the perception that these footprinted regions nearly overlap the DNA topoisomerase I cleavage sites that we have determined previously [also reported in Figure 2B, (13)]. In addition, we have clarified the absence of interference of *SIR2* and *NET1* in Fob1p-binding, observing that the Fob1p footprint, in *sir2Δ* and *net1Δ* strains, is similar to the one shown by the WT cells. Given the proximity between DNA topoisomerase I cleavage sites in the enhancer region and the binding sites of Fob1p, we asked the following question: are the cleavage sites produced by the DNA topoisomerase I in the NTS region due to the presence of the *FOB1* gene, or do they depend on the presence/absence of potential partners like *SIR2* and *NET1*?. When the study of the cleavage sites was conducted in strains mutant for *SIR2*, *NET1* or *FOB1* respectively, we observed, on the NTS1 region, a clear dependence on *FOB1* of the cleavages due to DNA topoisomerase I (Figure 3A). This represents the first observation in which the dependence of these sites upon a specific gene is reported. We hypothesize that a physical interaction, if any, between Fob1p and DNA topoisomerase I could be weak; in fact, previous reports (32) demonstrated that DNA topoisomerase I interacts only with two gene products, *TOF1* and *TOF2*, strongly enough to be detected in an extensive two-hybrids screening. When a Δ to f2 mutant was analysed, we observed that the cleavage efficiency was not affected (data not shown) suggesting that TOF2, described to be present in the RENT complex (33), does not interfere with the DNA topoisomerase I cleavage activity on the NTS1 sequence.

In a previous study (13) we found that the absence of transcription does not interfere with the capability of DNA topoisomerase I to cleave DNA nearby the 35S RNA promoter region. Actually, in the promoter region, where a specific cleavage site was also shown (13), *FOB1* dependence was not observed, (Figure 3B). This observation confirms the reported data (8) that consider the existence of two different mechanisms acting in the recruitment of silencing proteins on rDNA: one depending on Fob1p, Sir2p and Net1p contacting the enhancer region and the transcriptional silencing; the other involving components acting in a specific manner on rDNA, probably recruiting RNA polymerase I, as reported previously (34,35). Our observation implies that these complexes could

contain the DNA topoisomerase I activity, probably weakly bound and not detected by biochemical assays. The role of DNA topoisomerase I in this region does not seem to be related to rDNA replication, nor to rDNA transcription. In fact, when we analysed the DNA replication fork blocking activity on the RFBs and the RNA transcription (both of them proposed as important events for the induction of DNA recombination, see (36) we could not find any correlation between these processes and the possible production of alternative DNA topoisomerase I sites, as reported in Figure 4A and B.

Thus the DNA topoisomerase I cleavage production does not depend on replication and transcription of rDNA. Since we demonstrated that the cleavage sites in the enhancer region are *FOB1* dependent, we consider that the most reasonable explanation relies on the binding of Fob1p itself to DNA and on the possible consequent distortion of the double helix due to its binding. In order to verify the involvement of the chromosomal context on the cleavage efficiency of DNA topoisomerase I on NTS1 sequence, we analysed the cleavage profile induced by CPT addition in a rdnΔΔ strain (27) (lacking the whole chromosomal rDNA array) and containing the pNOY373 plasmid (carrying an entire rDNA unit). The results exclude an involvement of the chromosomal context because the ectopic copy of rDNA was cleaved by DNA topoisomerase I with an efficiency comparable to that one shown by the WT strain. Actually, it has been reported that bent DNA represents an efficient substrate for DNA topoisomerase I (37) and a similar deviation from linearity of the double helix could be exerted by Fob1p by binding the DNA. In a previous paper (9) an observation was reported, compatible with the wrapping of DNA around Fob1p, structure that may produce a significant bent in the DNA molecule that DNA topoisomerase I can recognize and efficiently cleave. This implies that the nicks produced by DNA topoisomerase I may be the starting points of *FOB1* mediated recombination events. Conversely, the hyper-rec phenotype observed in *top1Δ* strains (15) could be due to a different mechanism triggered by the accumulation of superhelical stress due to lack of the swivel activity of DNA topoisomerase I.

It is noteworthy that Net1p and Sir2p associate to DNA both in the enhancer and in the promoter regions (8). These associations are *FOB1* dependent at the enhancer and *FOB1* independent at the promoter. Interestingly, also the DNA topoisomerase I site-specific behaviour, reported in this paper, turns out to be *FOB1* dependent at the enhancer and independent at the promoter (Figure 3). Taken together these data suggest a possible involvement of DNA topoisomerase I in the RENT complex with Net1p, Sir2p and CdC14p (25). Given that Net1p shares significant sequence similarity (32) with Tof2 [Topoisomerase interacting factor 2; (32)] of *S.cerevisiae*, we hypothesize a possible interaction between Net1p and DNA topoisomerase I according to the previous idea that DNA topoisomerase I is also part of the RENT complex.

It has been recently reported that ribosomal DNA shows double strand breaks or nicked forms at positions overlapping those we found to be DNA topoisomerase I cleavage sites, and the authors of this work reported these ruptures to be *FOB1* dependent (38). We strongly suppose that the nicks reported in those experiments are actually the cleavage sites produced by DNA topoisomerase I that we found in this and a previous paper (13); in addition we observed also the capability of

top3Δ strain to produce the same pattern profile of WT strain (data not shown). For this reason we may exclude the involvement of DNA topoisomerase III activity [hypothesized in (13)] for the cleavage production in the regions analysed in our study.

The finding of an involvement of Fob1p in the site-specific activity of DNA topoisomerase I contributes to clarify the role of this enzyme in important events occurring in the rDNA such as the control of genome stability, transcriptional silencing and cellular aging.

REFERENCES

1. Petes,T.D. (1979) Yeast ribosomal genes are located on chromosome XII. *Proc. Natl. Acad. Sci. USA*, **76**, 410–414.
2. Nomura,M. (2001) Ribosomal RNA genes, RNA polymerases, nucleolar structures, and synthesis of rRNA in the yeast *Saccharomyces cerevisiae*. *Cold Spring Harb. Symp. Quant. Biol.*, **66**, 555–565.
3. Kulkens,T., Riggs,D.L., Heck,J.D., Planta,R.J. and Nomura,M. (1991) The yeast RNA polymerase I promoter: ribosomal DNA sequences involved in transcription initiation and complex formation *in vitro*. *Nucleic Acids Res.*, **19**, 5363–5370.
4. Keys,D.A., Lee,B.S., Dodd,J.A., Nguyen,T.T., Vu,L., Fantino,E., Burson,L.M., Nogi,Y. and Nomura,M. (1996) Multiprotein transcription factor UAF interacts with the upstream element of the yeast RNA polymerase I promoter and forms a stable preinitiation complex. *Genes Dev.*, **10**, 887–903.
5. Vogelauer,M., Cioci,F. and Camilloni,G. (1998) DNA–protein interactions at the *Saccharomyces cerevisiae* 35S rRNA promoter and its surrounding region. *J. Mol. Biol.*, **275**, 197–209.
6. van der Sande,C.A., Kulkens,T., Kramer,A.B., de Wijs,I.J., van Heerikhuizen,H., Klootwijk,J. and Planta,R.J. (1989) Termination of transcription by yeast RNA polymerase I. *Nucleic Acids Res.*, **17**, 9127–9146.
7. Brewer,B.J. and Fangman,W.L. (1988) A replication fork barrier at the 3′ end of yeast ribosomal RNA genes. *Cell*, **55**, 637–643.
8. Huang,J. and Moazed,D. (2003) Association of the RENT complex with nontranscribed and coding regions of rDNA and a regional requirement for the replication fork block protein Fob1 in rDNA silencing. *Genes Dev.*, **17**, 2162–2176.
9. Kobayashi,T. (2003) The replication fork barrier site forms a unique structure with Fob1p and inhibits the replication fork. *Mol. Cell. Biol.*, **23**, 9178–9188.
10. Kobayashi,T. and Horiuchi,T. (1996) A yeast gene product, Fob1 protein, required for both replication fork blocking and recombinational hotspot activities. *Genes Cells.*, **1**, 465–474.
11. Kobayashi,T., Heck,D.J., Nomura,M. and Horiuchi,T. (1998) Expansion and contraction of ribosomal DNA repeats in *Saccharomyces cerevisiae*: requirement of replication fork blocking (Fob1) protein and the role of RNA polymerase I. *Genes Dev.*, **12**, 3821–3830.
12. Defossez,P.A., Prusty,R., Kaeberlein,M., Lin,S.J., Ferrigno,P., Silver,P.A., Keil,R.L. and Guarente,L. (1999) Elimination of replication block protein Fob1 extends the life span of yeast mother cells. *Mol. Cell*, **3**, 447–455.
13. Vogelauer,M. and Camilloni,G. (1999) Site-specific *in vivo* cleavages by DNA topoisomerase I in the regulatory regions of the 35 S rRNA in *Saccharomyces cerevisiae* are transcription independent. *J. Mol. Biol.*, **293**, 19–28.
14. Bryk,M., Banerjee,M., Murphy,M., Knudsen,K.E., Garfinkel,D.J. and Curcio,M.J. (1997) Transcriptional silencing of Ty1 elements in the RDN1 locus of yeast. *Genes Dev.*, **11**, 255–269.
15. Christman,M.F., Dietrich,F.S. and Fink,G.R. (1988) Mitotic recombination in the rDNA of *S.cerevisiae* is suppressed by the combined action of DNA topoisomerases I and II. *Cell*, **55**, 413–425.
16. Zhu,J. and Schiestl,R.H. (2004) Human topoisomerase I mediates illegitimate recombination leading to DNA insertion into the ribosomal DNA locus in *Saccharomyces cerevisiae*. *Mol. Genet. Genomics*, **271**, 347–358.
17. Sherman,F., Fink,G.R. and Lawrence,C. (1983) *Methods in Yeast Genetics*. Cold Spring Harbor University Press, Cold Spring Harbor, N.Y.
18. Venditti,S. and Camilloni,G. (1994) *In vivo* analysis of chromatin following nystatin-mediated import of active enzymes into *Saccharomyces cerevisiae*. *Mol. Gen. Genet.*, **242**, 100–104.
19. Wu,C. (1980) The 5′ ends of *Drosophila* heat shock genes in chromatin are hypersensitive to DNase I. *Nature*, **286**, 854–860.
20. Almer,A. and Horz,W. (1986) Nuclease hypersensitive regions with adjacent positioned nucleosomes mark the gene boundaries of the PHO5/PHO3 locus in yeast. *EMBO J.*, **5**, 2681–2687.
21. Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) Molecular Cloning, 2nd Ed. (Cold Spring Harbor University Press, Cold Spring Harbor).
22. Bordi,L., Cioci,F. and Camilloni,G. (2001) *In vivo* binding and hierarchy of assembly of the yeast RNA polymerase I transcription factors. *Mol. Biol. Cell.*, **12**, 753–760.
23. Maxam,A.M. and Gilbert,W. (1977) A new method for sequencing DNA. *Proc. Natl Acad. Sci. USA*, **74**, 560–564.
24. Hsiang,Y.H., Hertzberg,R., Hecht,S. and Liu,L.F. (1985) Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. *J. Biol. Chem.*, **260**, 14873–14878.
25. Shou,W., Seol,J.H., Shevchenko,A., Baskerville,C., Moazed,D., Chen,Z.W., Jang,J., Shevchenko,A., Charbonneau,H. and Deshaies,R.J. (1999) Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex. *Cell*, **97**, 233–244.
26. Wang,J.C. (1996) DNA topoisomerases. *Annu. Rev. Biochem.*, **65**, 635–692.
27. Wai,H.H., Vu,L., Oakes,M. and Nomura,M. (2000) Complete deletion of yeast chromosomal rDNA repeats and integration of a new rDNA repeat: use of rDNA deletion strains for functional analysis of rDNA promoter elements *in vivo*. *Nucleic Acids Res.*, **28**, 3524–3534.
28. Tanny,J.C., Kirkpatrick,D.S., Gerber,S.A., Gygi,S.P. and Moazed,D. (2004) Budding yeast silencing complexes and regulation of Sir2 activity by protein–protein interactions. *Mol. Cell. Biol.*, **24**, 6931–6946.
29. Hereford,L.M. and Hartwell,L.H. (1974) Sequential gene function in the initiation of Saccharomyces cerevisiae DNA synthesis. *J. Mol. Biol.*, **84**, 445–461.
30. Thuriaux,P., Mariotte,S., Buhler,J.M., Sentenac,A., Vu,L., Lee,B.S. and Nomura,M. (1995) Gene RPA43 in *Saccharomyces cerevisiae* encodes an essential subunit of RNA polymerase I. *J. Biol. Chem.*, **270**, 24252–24257.
31. Nogi,Y., Yano,R. and Nomura,M. (1991) Synthesis of large rRNAs by RNA polymerase II in mutants of *Saccharomyces cerevisiae* defective in RNA polymerase I. *Proc. Natl Acad. Sci. USA*, **88**, 3962–3966.
32. Park,H. and Sternglanz,R. (1999) Identification and characterization of the genes for two topoisomerase I-interacting proteins from *Saccharomyces cerevisiae*. *Yeast*, **15**, 35–41.
33. Straight,A.F., Shou,W., Dowd,G.J., Turck,C.W., Deshaies,R.J., Johnson,A.D. and Moazed,D. (1999) Net1, a Sir2-associated nucleolar protein required for rDNA silencing and nucleolar integrity. *Cell*, **97**, 245–256.
34. Buck,S.W., Sandmeier,J.J. and Smith,J.S. (2002) RNA polymerase I propagates unidirectional spreading of rDNA silent chromatin. *Cell*, **111**, 1003–1014.

35. Cioci,F., Vu,L., Eliason,K., Oakes,M., Siddiqi,I.N. and Nomura,M. (2003) Silencing in yeast rDNA chromatin: reciprocal relationship in gene expression between RNA polymerase I and II. *Mol. Cell*, **12**, 135–145.

36. Rothstein,R., Michel,B. and Gangloff,S. (2000) Replication fork pausing and recombination or 'gimme a break'. *Genes Dev.*, **14**, 1–10.

37. Caserta,M., Amadei,A., Di Mauro,E. and Camilloni,G. (1989) *In vitro* preferential topoisomerization of bent DNA. *Nucleic Acids Res.*, **17**, 8463–8474.

38. Burkhalter,M.D. and Sogo,J.M. (2004) rDNA enhancer affects replication initiation and mitotic recombination: Fob1 mediates nucleolytic processing independently of replication. *Mol. Cell*, **15**, 409–421.