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Inactivation of topoisomerases affects transcription-dependent chromatin transitions in rDNA but not in a gene transcribed by RNA polymerase II

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Previous studies on a chromatin reporter gene (GAL–URARIB) in yeast showed that nucleosomes were maintained but rearranged during transcription in galactose, which was consistent with a local dissociation of histones at the site of the RNA polymerase. Furthermore, repositioning of nucleosomes occurred rapidly after glucose repression. Because nucleosomal disruption and transcription produce topological changes in the chromatin substrate, the effect of topoisomerase activity was tested by the insertion of GAL–URARIB in topoisomerase mutant strains. The chromatin structure was analysed by nuclease digestion and psoralen crosslinking, and compared with that of the rDNA locus. In GAL–URARIB, neither the inactivation of topoisomerases I, II or I and II generated nucleosomal loss during transcription, nor was topoisomerase activity required for repositioning of the nucleosomes after repression. In contrast, the inactivation of topoisomerase I promoted an enhanced psoralen accessibility of the transcribed rDNA, possibly because of altered supercoiling, and the inactivation of topoisomerases I and II disrupted the chromatin structure of the whole rDNA locus by redistribution of the nucleosomes. The inactivation of topoisomerase II alone had no effect. These observations substantiate a differential participation of topoisomerases in the modulation of the chromatin structures of rDNA genes and of a single copy polymerase II gene. It is suggested that topological stress in genes transcribed by RNA polymerase II might diffuse away into flanking regions.
Keywords: chromatin structure/RNA polymerases/topoisomerases/transcription/yeast

Introduction

Eukaryotic DNA is folded into two left-handed superhelical turns around octamers of histone proteins, to form an array of nucleosomes which is further condensed into chromatin fibres and higher order structures. Structural changes are required to provide access to the DNA for the initiation and elongation of transcription and to regenerate the inactive chromatin structure after transcription is turned off. Promoter accessibility may be provided by factors which efficiently compete with histones for DNA binding immediately after replication or which may mediate the dissolution or modification of pre-existing nucleosomes (reviewed in Becker, 1994; Wallrath *et al.*, 1994; Wolffe, 1994; Kornberg and Lorch, 1995). The mechanisms by which RNA polymerases manage to transcribe through nucleosomes are not known. It is sterically impossible for the polymerase to follow the DNA double-helix when it is folded on the histone octamer surface. Extreme models suggest the unfolding of nucleosomes or the dissociation of the histone octamer before, and reassembly behind, the RNA polymerase (reviewed in Thoma, 1991; Van Holde *et al.*, 1992). The most recent *in vitro* transcription experiments support a full displacement of the nucleosomes by RNA polymerase (Odonohue *et al.*, 1994; Studitsky *et al.*, 1994; reviewed in Lewin, 1994; Kornberg and Lorch, 1995). For many genes transcribed by RNA polymerase II *in vivo*, it has been shown quite convincingly that nucleosomes are present on transcribed DNA both immediately before and immediately after transcription, while a few other examples support a loss of nucleosomes or an altered nucleosomal structure (reviewed in Reeves, 1984; Thoma, 1991; Van Holde *et al.*, 1992). A local displacement of nucleosomes and a rapid reassembly behind the polymerase, leading to rearranged nucleosomes, was supported by studies on galactose-regulated genes in yeast (Cavalli and Thoma, 1993). In contrast, the transcription of ribosomal RNA genes by RNA polymerase I generates a loss of nucleosomes (Sogo *et al.*, 1984; Conconi *et al.*, 1989, 1992; Dammann *et al.*, 1993). Hence, in view of these observations, it is likely that the presence of nucleosomes on transcribed genes *in vivo* depends on many parameters, including the RNA polymerase, the rate of transcription, sequence-specific differences in histone binding or the chromosomal locus (Thoma, 1991).

Each nucleosome stores one negative supercoil (Keller, 1975; Simpson *et al.*, 1985), and transcription elongation promotes a build up of positive and negative supercoils in front and behind the RNA polymerase, respectively (Liu and Wang, 1987; Giaever and Wang, 1988). Hence, an additional parameter regulating nucleosomal stability may be the transcription-dependent local topological stress. The differential binding of histone octamers to positively and negatively supercoiled DNA has suggested that these supercoils might help to dissociate and reassemble histone octamers in the vicinity of the polymerase (Clark and Felsenfeld, 1991; Patterton and Vonholt, 1993). Other reconstitution experiments have supported an altered nucleosomal structure on positive supercoiled DNA and differences in the DNA binding of H3–H4 and H2A–H2B, and suggested a model in which H3–H4 remain attached to DNA during passage of the polymerase

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(Jackson, 1993; Jackson *et al.*, 1994). Because a nucleosome folds ~160 bp, the passage of a polymerase will produce more supercoils than required to destabilize the nucleosomes (Thoma, 1991). This local superhelical stress may be removed by topoisomerase activity coupled with RNA polymerases (Stewart *et al.*, 1990).

The inactivation of topoisomerases I and II in yeast *Saccharomyces cerevisiae* (*top1 top2^{ts}* mutant) showed that the transcription of rDNA by RNA polymerase I was more severely affected than transcription by RNA polymerase II. In strains with deleted topoisomerase I, topoisomerase II may substitute for a loss of topoisomerase I activity (Brill *et al.*, 1987; Brill and Sternglanz, 1988). Topoisomerase inactivation favours the initiation but prevents the elongation of rDNA transcription (Schultz *et al.*, 1992). The inactivation of topoisomerases I and II resulted in a transcription-dependent accumulation of negative supercoils on plasmid DNA, possibly because positive supercoils were removed by an unknown topoisomerase (Brill and Sternglanz, 1988). On the other hand, transcription-dependent positive supercoils were produced artificially on plasmids by the expression of *Escherichia coli* DNA topoisomerase I, which selectively removes negative supercoils in a *top1 top2^{ts}* mutant (Giaever and Wang, 1988). These positive supercoils were reported to disrupt the chromatin structure on yeast plasmid DNA (Lee and Garrard, 1991). While it may be assumed that supercoils are also generated in genes transcribed in the genome, their effects on the chromatin structure are not known.

Recently we have constructed and characterized an artificial gene called GAL–URARIB (Figure 1A) which is suited to monitoring transcription-dependent chromatin transitions after the activation or repression of transcription. It is regulated by the GAL1 promoter, repressed in glucose and heavily transcribed by RNA polymerase II in galactose. It shows positioned nucleosomes in the 5′ region in the inactive state, no nucleosomal loss but rearranged nucleosomes during transcription, as measured by psoralen crosslinking and indirect end labelling. In addition, it manifests a rapid regeneration of the inactive structure after the repression of transcription by glucose (Cavalli and Thoma, 1993). By inserting GAL–URARIB in the appropriate topoisomerase mutant strains, we demonstrate here that transcription-dependent chromatin transitions can still occur irrespective of the inactivation of topoisomerases, but the inactivation of topoisomerase I or I and II dramatically alters the chromatin structure of the rDNA locus.

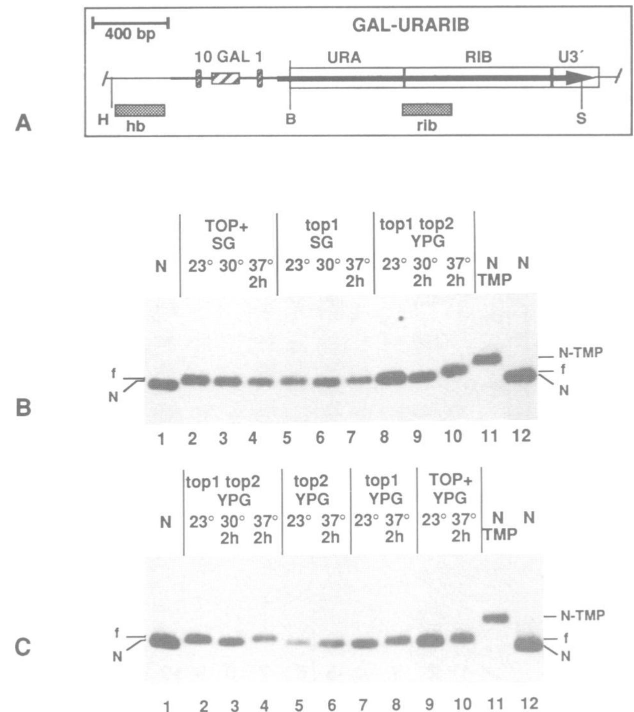

Fig. 1. Nucleosomes are maintained in transcribed GAL–URARIB despite topoisomerase inactivation. (**A**) The GAL–URARIB gene integrated in the LEU2 locus of chromosome III. The following elements are indicated. The GAL1 promoter (GAL1) with the upstream activating sequence (hatched box) and the flanking TATA boxes; a sequence of the URA3 gene (URA); an rDNA sequence of *Dictyostelium discoideum* (RIB); the 3′ end of URA3 (U3′). Probes are shown as dark boxes (hb, rib). Arrows indicate the direction of transcription. Relevant restriction sites are: *Hin*dIII (H), *Bam*HI (B) and *Sma*I (S). (**B**) Psoralen analysis of GAL–URARIB in *TOP$^+$* cells and in *top1*, *top2^{ts}* or *top1 top2^{ts}* mutants. FTY105 (*TOP$^+$*) or FTY112 (*top1*) were grown in minimal medium containing galactose (SG) at 23 (lanes 2 and 5) or 30°C (lanes 3 and 6). An aliquot was shifted to 37°C in SG for 2 h (2 h; lanes 4 and 7). FTY111 (*top1 top2^{ts}*) was grown in complex galactose medium (YPG) at 23°C (lane 8). Aliquots were shifted for 2 h to 30 and 37°C (lanes 9 and 10). Cells were crosslinked with trimethylpsoralen on ice. The DNA was purified, digested with *Bam*HI and *Sma*I, run on an agarose gel, blotted and hybridized to the rib probe. Migration of the bands was compared with naked DNA not crosslinked (N) or crosslinked with trimethylpsoralen *in vitro* (N-TMP). Under all conditions, only one band (f-band, nucleosomal band) was detected. (A non-nucleosomal band would run close to the N-TMP band; see ribosomal genes in Figure 3.) (**C**) An independent set of experiments was performed on cells grown in complex galactose medium (YPG).

Results

DNA topoisomerase inactivation has no effect on nucleosomal stability during the transcription of GAL–URARIB by RNA polymerase II

To test whether the inactivation of topoisomerases might affect nucleosomal stability during transcription or the regeneration process after repression, GAL–URARIB was integrated into the LEU2 locus of topoisomerase mutants. FTY105 (*TOP1 TOP2*) is the control strain with functional topoisomerases (referred to as TOP$^+$); in FTY112 (*top1*), the gene for topoisomerase I is disrupted; in FTY107 (*top2^{ts}*), topoisomerase II is temperature sensitive and inactivated at 37°C (DiNardo *et al.*, 1984; Giaever and Wang, 1988); FTY111 (*top1 top2^{ts}*) contains the disrupted topoisomerase I gene and the temperature-sensitive topoisomerase II mutation.

To test for nucleosomal loss during transcription, a psoralen-dependent gel retardation assay was used. Trimethylpsoralen preferentially crosslinks double-stranded DNA in the linker between nucleosomes and in non-nucleosomal regions, while DNA in nucleosomes is not affected. The binding of psoralen leads to a retardation of DNA during electrophoresis. Nucleosomal substrates lead to a weak retardation (called fast band, or f-band), while non-nucleosomal substrates, such as transcribed ribosomal genes, lead to a strong retardation (called slow band, or s-band; Conconi *et al.*, 1989; Cavalli and Thoma, 1993; Dammann *et al.*, 1993).

The yeast cultures were grown in galactose medium

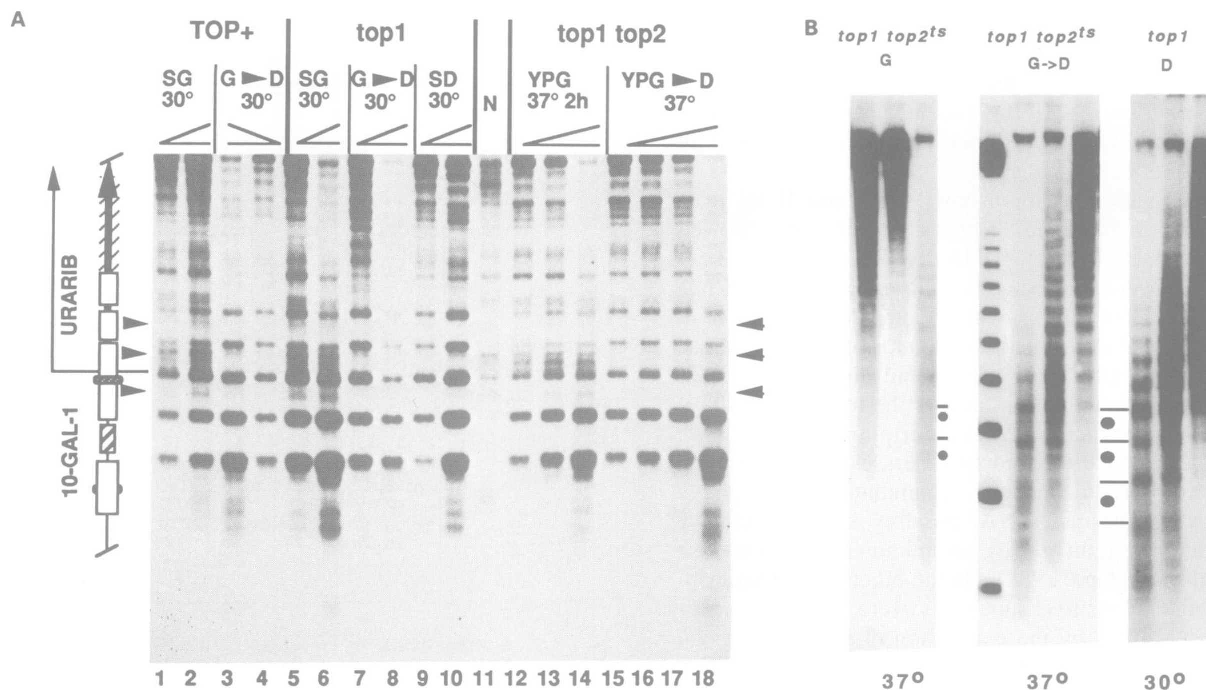

Fig. 2. Transcription-dependent loss of nucleosomal positioning and rapid repositioning after glucose repression are independent of topoisomerase activity. (**A**) Nucleosomal positioning on GAL–URARIB was analysed in FTY105 cells (*TOP*$^+$) and FTY112 (*top1*) or FTY111 (*top1 top2*^{ts}) mutants by MNase digestion and indirect end labelling. Chromatin (lanes 1–10 and 12–18) and naked DNA (lane 11) were digested with different amounts of MNase (>,<). The DNA was purified, restricted with *Hin*dIII, run on an agarose gel, blotted and hybridized to the probe hb (Figure 1A). Protection of the cutting sites in chromatin (arrow heads) within 140–200 bp was interpreted as positioned nucleosomes. The non-nucleosomal GAL1–10 UAS (hatched box), GAL1 and GAL10 TATA boxes (hatched ovals), and the transcript directed by the GAL1 promoter (thin arrow) are shown. *TOP*$^+$ and *top1* cells were grown at 30°C in SG medium, and shifted to glucose SD for 10 min (G > D) to repress GAL–URARIB transcription. *top1* cells grown in SD medium are shown in lanes 9 and 10. *top1 top2* cells were grown at 23°C in YPG and incubated for 2 h at 37°C to inactivate topoisomerase II (lanes 12–14). Half of the cells were further shifted to YPD (at 37°C) for 10 min (lanes 15–18). Positioned nucleosomes (open boxes) were observed on the promoter and the 5′ end of the coding region of GAL–URARIB in glucose (lanes 9 and 10). In all cases, positions were lost in galactose and positioning was largely re-established after 10 min in glucose. (**B**) Nucleosomal repeat patterns are shown for *top1* cells grown in glucose (D) and *top1 top2*^{ts} cells incubated at 37°C in YPG (G) and 10 min in glucose (G → D). The digestion patterns generated by MNase were hybridized to the rib probe (Figure 1A). Dashes indicate the nucleosomal repeat characteristic for cells grown in glucose; dots indicate additional bands of an altered repeat in G and G → D lanes. The nucleosomal repeat pattern is not completely regenerated after 10 min of glucose repression in *top1 top2*^{ts} at 37°C. A 256 bp ladder is included as a size marker.

(SG or YPG) at 23 or 30°C, and shifted to a semipermissive (30°C) or nonpermissive temperature (37°C) for 2 h to inactivate the temperature-sensitive topoisomerase II (DiNardo *et al.*, 1984; Brill and Sternglanz, 1988; Giaever and Wang, 1988). To avoid the temperature effects on psoralen crosslinking, cells were chilled on ice immediately prior to crosslinking. Only a nucleosomal band was observed in all strains and at all temperatures (Figure 1B and C). A non-nucleosomal s-band, which would run close to the N-TMP marker (lanes 11; see also rDNA of the same samples, Figure 3), was not detected. The transcription of galactose-regulated genes was not affected in *top1*, *top2*^{ts} or *top1 top2*^{ts} mutants (Brill and Sternglanz, 1988; Gartenberg and Wang, 1992; see also nucleosomal rearrangement in Figure 2). Therefore our results show that neither the absence of topoisomerase I nor a defective topoisomerase II, nor a combination of the two, were sufficient to destabilize the nucleosomes during transcription and to generate substantial nucleosomal loss. Furthermore, this effect did not depend on whether cells were grown in minimal or full medium (compare *TOP*$^+$ and *top1* in Figure 1B and C), and high temperature (37°C) did not promote nucleosomal loss.

We noted that the migration of the f-band was slightly variable. In particular, the DNA band of the double mutant FTY111 (*top1 top2*^{ts}) was slightly more retarded at 37°C (Figure 1B, lane 10). This effect was less pronounced in a second independent experiment (Figure 1C, compare lane 2 with lane 4). The resolution of the psoralen technique and gels, as used here, does not allow us to decide whether this effect reflects a slightly enhanced psoralen accessibility in chromatin or whether it is attributed to a difference in psoralen penetration and crosslinking.

Topoisomerases are not required for repositioning of the nucleosomes after glucose repression

Strains FTY105 (*TOP*$^+$) and FTY112 (*top1*) were analysed for nucleosomal positioning during transcription in galactose and 10 min after glucose repression at 30°C (Figure 2). FTY111 (*top1 top2*^{ts}) was analysed in galactose after a 2 h shift to 37°C and after glucose repression at 37°C. (Special care was given to maintain the temperature at 37°C during chromatin preparation up to lysis of the spheroplasts.) Chromatin and deproteinized control DNA were digested with micrococcal nuclease (MNase), and the cutting sites were displayed by indirect end labelling.

In galactose medium, the band pattern created by MNase was similar to that created in deproteinized DNA, and no significant differences were observed between the different strains (compare lanes 1, 2, 5, 6 and 12–14 with lane 11).

The pattern was also indistinguishable from that of GAL–URARIB transcribed in strain GCY5 (Cavalli and Thoma, 1993). Because a psoralen analysis showed no remarkable loss of nucleosomes (Figure 1), the nucleosomes must be present in the transcribed genes but rearranged and not precisely positioned.

When chromatin was analysed 10 min after glucose repression, clear protection against MNase was observed in the 5′ end of the gene, consistent with an array of positioned nucleosomes (Figure 2, lanes 3, 4, 7, 8 and 15–18). This effect is most obvious for the second nucleosome (middle arrow) which protects two strong cutting sites. The nucleosomal arrangement was similar to that in cells which were grown in glucose and had never been in galactose (lanes 9 and 10), and similar to the pattern observed in GCY5 after 10 min of glucose repression (Cavalli and Thoma, 1993).

A nucleosomal repeat analysis in GCY5 showed a clear repeat in glucose and a smeary altered repeat pattern in galactose. Although a major fraction of chromatin regenerated nucleosomal positions after 10 min of glucose repression, complete regeneration of the nucleosomal repeat required 2.5 h (Cavalli and Thoma, 1993). Figure 2B shows a characteristic nucleosomal repeat pattern when cells are grown in glucose (Figure 2B, *top1*). When *top1 top2*^{ts} cells were grown in galactose the pattern was smeary, with some residual bands consistent with rearranged nucleosomes (dots). After 10 min of glucose repression, a major fraction of bands was observed which corresponds to the repeat of glucose-grown chromatin, while a minor population of additional bands (dots) documented that regeneration was not yet complete. A seeming contradiction between almost complete protection in the second nucleosome (Figure 2A) and a minor fraction of unusual repeats (dots, Figure 2B) may relate to the rib probe which detects chromatin arrangement downstream of the positioned nucleosomes. This observation suggests that rearrangement in the 5′ region could be faster than in the 3′ region.

In summary, the results support the following conclusions. (i) The chromatin structures of GAL–URARIB are indistinguishable in different strains. (ii) The rearranged chromatin structure observed in galactose-grown cells demonstrates that the GAL–URARIB genes were transcribed. (iii) Neither the transcription-dependent rearrangement of nucleosomes nor the rapid regeneration of the inactive structure observed 10 min after repression require topoisomerase activity. (iv) Because the *top1 top2*^{ts} host strain RS192 (from which FTY111 was derived) ceases DNA replication upon shifting to the nonpermissive temperature (Brill *et al.*, 1987), the chromatin regeneration in FTY111 does not depend on DNA replication.

Effects of DNA topoisomerase inactivation on chromatin structure of rDNA genes

The elongation of rDNA transcription is severely inhibited in *top1 top2*^{ts} double mutants, but not in *top1* and *top2*^{ts} single mutants (Schultz *et al.*, 1992). Psoralen crosslinking (Figure 3) and MNase digestions (Figure 4) show effects of topoisomerase inactivation on the chromatin structure of the rDNA genes.

For the psoralen analysis (Figure 3), the same DNA was used as for Figure 1. DNA was cut with *Eco*RI, run on agarose gels, blotted and hybridized to the XX probe (Figure 3A), which detects a smaller 1.9 kb (bottom bands in Figure 3B–D) and a longer 2.8 kb fragment (top bands in Figure 3B–D). The smallest fragment located between the 1.9 and the 2.8 kb fragments (Figure 3A) runs off the gel. In FTY105 (TOP$^+$), a strong nucleosomal band was observed for each fragment at all temperatures (f-bands in Figure 3B, lanes 2–4). A minor fraction was retarded (s-band) and represented transcribed ribosomal genes that lost nucleosomes (Dammann *et al.*, 1993). Hence, a temperature shift to 37°C produced no additional loss of

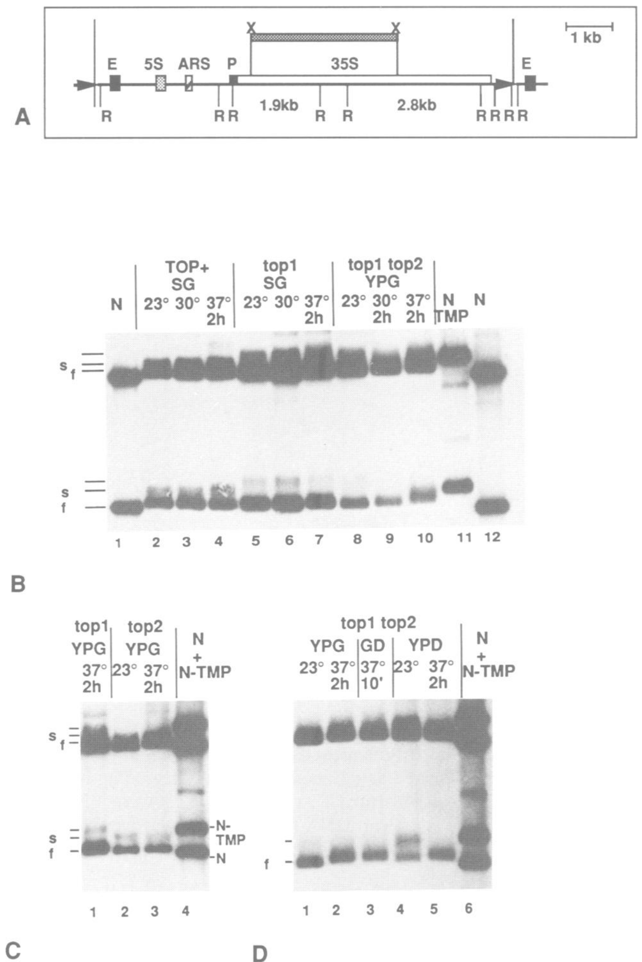

Fig. 3. Topoisomerase inactivation affects the chromatin structure of the rDNA. (**A**) One unit of the repetitive rDNA. Indicated are the 5S ribosomal RNA gene (5S), the enhancer (E), the origin of replication (ARS), the promoter (P), a probe (dark box, XX), *Eco*RI restriction sites (R) and the 1.9 and 2.8 kb fragments. The arrow indicates the direction of transcription. (**B**) The analysis by psoralen crosslinking was performed as described in Figure 1. Conditions are indicated on top of the panels. FTY105 (*TOP*$^+$) and FTY107 (*top2*^{ts}) show strong f-bands and weak s-bands. FTY112 (*top1*) and FTY111 (*top1 top2*^{ts}) at 23°C produce a dominant f-band and a more pronounced smeary retardation of the s-band (lanes 5–7). (**C**) Conditions were: FTY112 (*top1*) in YPG at 23°C and shifted to 37°C for 2 h (lane 1; same as Figure 1C, lane 8); and FTY107 (*top2*^{ts}) in YPG at 23°C and shifted to 37°C for 2 h (lanes 2 and 3; same as Figure 1C, lanes 5 and 6). (**D**) Conditions for FTY111 (*top1 top2*^{ts}) were: YPG at 23°C and shifted to 37°C for 2 h, and afterwards to YPD for 10 min (lanes 1–3; lanes 1 and 2 are the same as used for Figure 1C, lanes 2 and 4); and YPD at 23°C and shifted to 37°C for 2 h (lanes 4 and 5). The data in (B), lanes 8–10, and in (D), lanes 1 and 2, are from independent experiments.

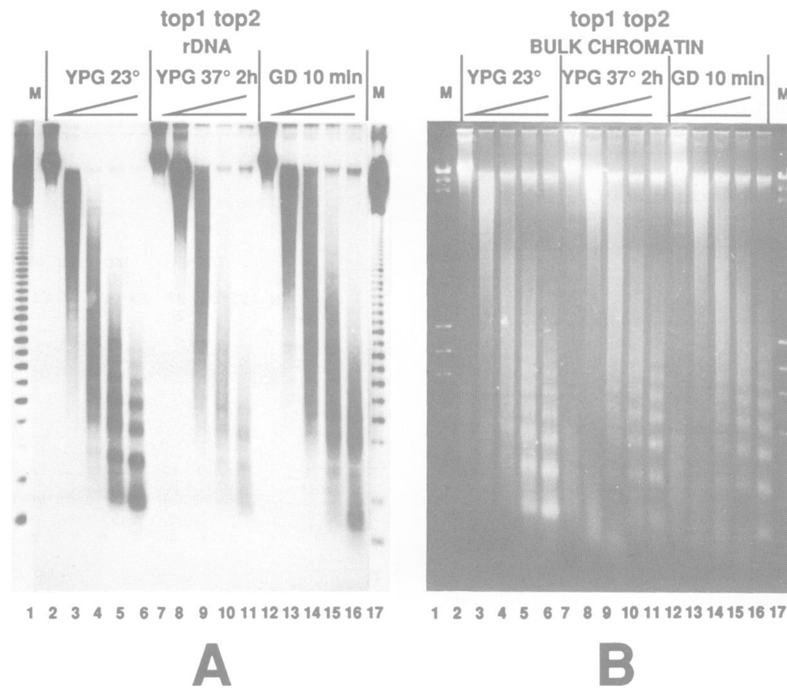

Fig. 4. Inactivation of topoisomerases I and II disrupts the nucleosomal arrangement in the coding region of the rDNA. Chromatin was digested with increasing concentrations of micrococcal nuclease (<), and the DNA fragments were analysed by gel electrophoresis. (**A**) The nucleosomal patterns in the rDNA were displayed using an XX probe of the coding region (Figure 3A). (**B**) The nucleosomal patterns of the genome were visualized by ethidium staining. Conditions for FTY111 (*top1 top2*^{ts}) were YPG at 23°C (lanes 2–6), shifted to 37°C for 2 h (lanes 7–11) and shifted to YPD for 10 min (lanes 12–16). A 123 bp DNA ladder (BRL) was used for calibration (A, lane 10). A mixture of 300 ng λ DNA (Boehringer) digested by *Hin*dIII and 300 ng ϕX DNA digested by *Hae*III (Boehriger) was used for the calibration (B, lanes 1 and 17).

nucleosomes in the rDNA locus, and nucleosomes were also lost during transcription at a low temperature (23°C).

When FTY112 (*top1*) was analysed (Figure 3B, lanes 5–7), most of the DNA was detected in the nucleosomal f-band. The second population displayed a smeary band-shift which was higher at all temperatures (supershift) when compared with the s-bands of *TOP*$^{+}$ cells. Apparently, the chromatin of active rDNA genes is more accessible to psoralen crosslinking in *top1* cells. This might reflect an increase in the superhelical density or an additional loss of proteins that restrict psoralen accessibility. In contrast to the *top1* mutant, the inactivation of topoisomerase II by a temperature shift from 23 to 37°C showed no altered psoralen crosslinking (and no supershift; Figure 3C, lanes 2 and 3), suggesting that topoisomerase II had no obvious effect on nucleosomal stability in transcribed rDNA.

In galactose medium at 23°C, FTY111 (*top1 top2*^{ts}) showed the same pattern as the *top1* cells, namely a nucleosomal f-band and a supershift consistent with an inactive topoisomerase I and an active topoisomerase II (Figure 3B, lane 8 and D, lane 1). When FTY111 (*top1 top2*^{ts}) was shifted for 2 h to 37°C to inactivate DNA topoisomerase II, only one band was detected with a mobility slightly slower than the nucleosomal f-band (Figure 3B, lane 10 and D, lane 2). An intermediate state was observed after 2 h at 30°C (Figure 3B, lane 9). The situation was much clearer when cells were grown in glucose medium, indicating that a larger fraction of the rDNA genes was actively transcribed in glucose (Figure 3D, lane 4). Upon shifting cells in YPD to 37°C for 2 h, a transition from two bands to one band migrating slightly higher than the nucleosomal band was observed (Figure 3D, lane 5). These data demonstrate that a structural transition occurs in the coding region of the ribosomal genes when topoisomerase I or topoisomerases I and II are inactivated. This transition may include a redistribution of the nucleosomes between previously active and inactive rDNA regions (see below).

As an alternative approach, the nucleosomal repeat pattern of the coding region of rDNA was analysed (Figure 4). To allow for a direct comparison between polymerase I and polymerase II genes, the conditions used (YPG) were the same as those employed to study transcription-dependent transition in GAL–URARIB. In FTY111 (*top1 top2*^{ts}), a smeary repeat was observed at the permissive temperature (23°C) in galactose (Figure 4A, lanes 2–6), similar to those seen in FTY105 (*TOP*$^{+}$) and FTY112 (*top1*) (results not shown). Consistent with the psoralen analysis (Figure 3), the nucleosomal repeat may represent the fraction of rDNA organized in nucleosomes, while the smeary background may be created by the non-nucleosomal fraction. However, after 2 h at 37°C, the repeat was lost in favour of a smear (Figure 4A, lanes 7–11). No fragments smaller than 150 bp were detected, although transfer was efficient, as seen by the transfer of the 123 bp marker DNA band (Figure 4A, lanes 1 and 17). Because psoralen crosslinking supports the presence of nucleosomes under these conditions, the smear is interpreted as irregularly spaced, rearranged or partially disrupted nucleosomes. Because the repeat pattern at a lower temperature and in the other strains reflected the inactive nucleosomal rDNA, the change into a smear shows that the chromatin structure of the inactive rDNA was affected.

In contrast to the rapid changes in chromatin structure, which were observed in GAL–URARIB after the addition of glucose, the smeary pattern of rDNA did not revert into a nucleosomal repeat (Figure 4A, lanes 12–16), nor was a change observed in psoralen crosslinking (Figure 3D, lane 3). Moreover, no differences in the bulk genomic repeat were observed at 23, 37°C and after the addition of glucose (Figure 4B). This shows that the disruption of the chromatin structure as a consequence of topoisomerase inactivation was specific for the rDNA cluster.

Discussion

Transcription is coupled to topological changes in DNA which may require topoisomerase activity. Because nucleosomes store one negative superhelical turn of DNA, unfolding as well as dissociation and reassociation of the nucleosomes will release or adsorb supercoils. In addition, positive and negative supercoils are produced in front and behind a moving RNA polymerase, respectively (Liu and Wang, 1987; Giaever and Wang, 1988). Studies with yeast topoisomerase mutants have shown that neither topoisomerase I nor topoisomerase II are essential for transcription by RNA polymerase II. However, plasmids carrying transcriptionally active genes were found to be extremely negatively supercoiled when isolated from mutants lacking topoisomerase I. It was suggested that the accumulation of negative supercoils was related to an unidentified activity which removes the positive supercoils, and that topoisomerase I is normally required to relax the transcriptionally induced supercoils (Brill and Sternglanz, 1988). Our results show that the inactivation of topoisomerase I in *top1* and *top1 top2^ts^* mutants leads to an increased psoralen accessibility in the s fraction of rDNA. We attribute this effect to the active genes, because the intensity of the nucleosomal f-band (inactive chromatin) was not affected. Because negative supercoiling was shown to increase the binding of psoralen (Sinden *et al.*, 1980) and the topoisomerase inactivation generates negative supercoils in yeast plasmids (Brill and Sternglanz, 1988), our data may indicate that the inactivation of topoisomerase I leads to a transcription-dependent increase of negative supercoiling in the rDNA locus. Another explanation for the altered psoralen accessibility could be the additional loss of nucleosomes, assuming that not all nucleosomes (histones) were displaced under normal conditions. Assuming that RNA polymerases may partially protect against psoralen crosslinking, the reduced loading of the genes with RNA polymerases could enhance psoralen binding. This explanation is less likely because *top1* mutants did not show a reduced rRNA synthesis (Brill *et al.*, 1987; Schultz *et al.*, 1992).

A more dramatic chromatin transition involving the rDNA locus was observed after the inactivation of topoisomerases I and II in *top1 top2^ts^* cells at 37°C. Under these conditions transcription elongation is strongly inhibited, presumably by the accumulation of supercoils (Brill and Sternglanz, 1988; Schultz *et al.*, 1992). The nucleosomal repeat changed into a smear, and psoralen crosslinking revealed only one band which migrated slightly slower than a normal nucleosomal band. This demonstrates that chromatin was disrupted throughout the locus, including those rDNA units that were inactive and organized in nucleosomes prior to the inactivation of topoisomerase II. One explanation is that the accumulation of negative supercoils in the whole rDNA locus caused a redistribution of the nucleosomes from inactive genes to the previously active ones. This was consistent with the slight increase in psoralen accessibility (slightly slower mobility). An additional loss of individual histones or some sort of unfolding of the nucleosomal structure, leading to a loss of periodicity by MNase pattern, may not be excluded.

Although a dramatic increase in the negative supercoiling of plasmid DNA carrying polymerase II genes was induced in *top1* and *top1 top2^ts^* mutants (Brill and Sternglanz, 1988), neither an altered psoralen accessibility nor an altered nucleosomal arrangement could be recorded on the chromosomal GAL–URARIB gene. This means that neither a destabilization nor a loss of nucleosomes could be induced. Nucleosomes were shown previously to be disrupted by the induction of positive supercoils on yeast plasmid DNA (Lee and Garrard, 1991). Because nucleosomes store negative supercoils, it is possible that nucleosomes on GAL–URARIB are stabilized even in the absence of topoisomerase activity, and that the reassociation of locally displaced nucleosomes could be facilitated. Alternatively, it may be considered that supercoils generated in polymerase II genes spread away into the flanking regions of the chromosome in such a way that a critical density of torsional stress is never achieved on the gene itself. This idea may be complemented by the observation that the inhibition of DNA topoisomerase II in a *Drosophila* cell line by novobiocin prevented the structural alterations (hypersensitive sites) which normally accompany heat induction in the hsp70 genes, as well as the re-establishment of the preinduced chromatin organization (Han *et al.*, 1985). These genes are apparently organized into chromatin domains with matrix attachment sites (reviewed in Gasser and Laemmli, 1987). In contrast to higher eukaryotes, yeast genes are short and placed next to each other without much of an intergenic spacer (Oliver, *et al.*, 1992); organization in functional domains is not firmly established (reviewed in Thoma *et al.*, 1993). Hence, it is conceivable that topological stress may diffuse away from yeast genes but may be retained in the domains of higher eukaryotes.

Regeneration of the inactive structure in GAL–URARIB after glucose repression was not affected by the inactivation of topoisomerases, not even in a *top1 top2^ts^* strain after 2 h at 37°C, when the chromatin structure in the rDNA locus was already heavily disrupted. Because DNA synthesis is inhibited efficiently under these conditions (Brill *et al.*, 1987), we infer that DNA replication is not needed for nucleosomal repositioning in GAL–URARIB. Hence, in this respect, regeneration resembles the reformation of nucleosomes after repression of the PHO5 promoter in yeast and the rat TAT gene, which take place in the absence of DNA replication (Reik *et al.*, 1991; Schmid *et al.*, 1992). A regeneration mechanism is only expected to need topoisomerase activity if nucleosomes are dissociated or unfolded during transcription and need to be reassembled or refolded. Therefore our results are consistent with the observation that nucleosomes were present but rearranged on the transcribed gene. Regeneration of the inactive structure could therefore occur by

repositioning of the nucleosomes by sliding or a rapid local dissociation and reassembly.

Materials and methods

Yeast strains

GCY5 [*MATα ura3-52 his3-200 ade2-101 lys2-801 tyr1-501 LEU2::YIpGC1(GAL–URARIB, URA3)*] has been described previously (Cavalli and Thoma, 1993). W303-1a (*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*), RS190 (*W303-1a top1-8::LEU2*), RS191 (*MATa ade2 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 top2-1*) and RS192 (*RS191 top1-8::LEU2*) were kindly provided by R.Sternglanz. The integration plasmid containing GAL–URARIB, YIpGC1 (Cavalli and Thoma, 1993), was linearized by Asp718 and integrated in LEU2 by transformation to give strains FTY105 [*W303-1a leu2-3,112:: YIpGC1(GAL–URARIB URA3)*], FTY112 [*RS190 leu2-3,112::YIpGC1 (GAL–URARIB URA3)*], FTY107 [*RS191 leu2-3,112::YIpGC1(GAL–URARIB URA3)*] and FTY111 [*RS192 leu2-3,112::YIpGC1(GAL–URARIB URA3)*]. Yeast cells were cultured in full medium containing glucose (YDP) or galactose (YPG), or in synthetic medium containing glucose (SD) or galactose (SG), according to Sherman *et al.* (1981).

Chromatin analysis by MNase

Cultures (1–3 l) were grown exponentially to an OD_{600} of 0.5–0.7. Media used were as indicated in the figure legends. For glucose repression, an aliquot of the galactose-grown culture was centrifuged at room temperature for 2 min at 5000 r.p.m. in a GS-3 rotor (Sorvall). The cell pellets were resuspended in prewarmed glucose medium and incubated for an additional 10 min. Cells were harvested by centrifugation, washed and converted to spheroplasts by Zymolyase (100 000 IU/g; Seikagaku Kogyo Co., Tokyo, Japan) at 30°C. Lysis of the spheroplasts, chromatin preparation, digestion with MNases, analysis of the nucleosomal repeats and mapping of the nucleosomal positions were performed as described previously (Bernardi *et al.*, 1991; Cavalli and Thoma, 1993). For experiments at 37°C, the appropriate cultures were shifted to 37°C by incubation in a water bath (50°C) for 1–2 min and incubated further by shaking at 37°C for 2 h. The *top2*^{ts} strains stopped growing. All steps up to spheroplasting were performed at 37°C.

Chromatin analysis by psoralen crosslinking

A method developed by Conconi *et al.* (1989) and adapted for yeast *S.cerevisiae* (Dammann *et al.*, 1993) was used with minor modifications. Cultures grown as described above. 5×10^8 cells were collected by centrifugation in a table-top centrifuge at the appropriate temperature (23, 30 and 37°C), resuspended in 50 ml prewarmed water, recollected by centrifugation, resuspended in 300 µl ice-cold TE (10 mM Tris–HCl, pH 7.6, 1 mM EDTA) and placed in multiwell plates (4 cm^2/well, Nunc) on ice. After the addition of 15 µl 4,5′,8-trimethylpsoralen (Sigma) stock solution (200 µg/ml in ethanol), the samples were incubated in the dark for 5 min and irradiated for 5 min with predominantly 366 nm light from a spot lamp (144 G5-100/SP; Mercuro Sylvana), in an irradiation apparatus B-100A,2A (Ultraviolet Inc.) at a distance of 6 cm. Psoralen addition and irradiation was repeated four times more. After psoralen crosslinking, cells were washed, converted to spheroplasts with Zymolyase at 23, 30 or 37°C and DNA was purified as described previously (Dammann *et al.*, 1993). For crosslinking of the control DNA, DNA was extracted from 10^9 cells and dissolved in 100 µl TE. To 50 µl of the DNA solution, 2.5 µl psoralen stock solution were added and crosslinking was performed as above, except that psoralen addition and crosslinking were carried out twice. For an analysis of the specific restriction fragments, 300–900 ng DNA were digested with the appropriate restriction enzymes (indicated in the figure legends) and run in 1.3% agarose gels (20×24 cm) in 50 mM Tris, 20 mM sodium acetate, 1.6 mM EDTA–disodium salt, pH 7.8, at 65 V for 17–18 h, and stained for 30 min in ethidium bromide (0.5 µg/ml). Psoralen crosslinking was reversed by irradiation of the gel with 254 nm light at a dose of 6 kJ/m^2 (Sylvana G15 T8). The gel was soaked for 15 min in 0.25 N HCl, rinsed in distilled water and blotted overnight in 0.4 N NaOH to Biodyne B (PALL) or Zeta-Probe (Bio-Rad) membranes. Prehybridization was performed in 0.25 M sodium phosphate, pH 7.2, 7% SDS, 1 mM EDTA, 50 µg/ml tRNA for 1 h at 68°C. Hybridization was carried out in 0.25 M sodium phosphate, pH 7.2, 7% SDS, 1 mM EDTA overnight at 68°C. Washing was performed twice for 45 min at 68°C with a solution of 40 mM sodium phosphate, pH 7.2, 5% SDS, 1 mM EDTA, then twice for 45 min at 68°C in 40 mM sodium phosphate, pH 7.2, 1% SDS, 1 mM EDTA.

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