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Nucleosomes represent a physical barrier for cleavage activity of DNA topoisomerase I *in vivo*.

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Running title: DNA topoisomerase I and nucleosomes

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SYNOPSIS

DNA topoisomerase I together with the other cellular DNA topoisomerases releases the torsional stress from DNA caused by processes such as replication, transcription and recombination. Despite the well defined knowledge of its mechanism of action, DNA topoisomerase I *in vivo* activity has been only partially characterized. In fact the basic question concerning the capability of the enzyme to cleave and rejoin DNA wrapped around histone octamer remains still unanswered.

By studying both *in vivo* and *in vitro* the cleavage activity of DNA topoisomerase I in the presence of camptothecin on a repeated trinucleotide sequence, $(TTA)_{35}$, lying in the chromosome XIII of *Saccharomyces cerevisiae*, we can conclude that nucleosomes represent a physical barrier for the enzyme activity.

Keywords: chromatin; DNA structure; DNA topoisomerase I; nucleosomes; S. cerevisiae; trinucleotide repeats.

INTRODUCTION

DNA topology in eukaryotic nuclei is controlled by a specific class of proteins: DNA topoisomerases [1]. These enzymes are classified into two groups depending on their catalytic activity: type I enzymes change DNA topology by breaking and rejoining one DNA strand at a time, while type II act contemporaneously on both strands. In eukaryotes, DNA topoisomerase IB reduces the torsional stress arising from basic DNA transactions like transcription, replication and recombination [2].

Although DNA topoisomerase I is involved in such important functions, the enzyme is not essential in *S. cerevisiae* [3].

Despite the well-known mechanisms of DNA topoisomerase I activity and the susceptibility to different inhibitors [4], its accessibility to the DNA *in vivo* has been poorly investigated. To date, evidences about the *in vivo* DNA topoisomerase I cleavage activity only refer to nucleosome free regions [5-7] and it is not known whether nucleosomes could represent a barrier for DNA topoisomerase I to access DNA. In fact it has been largely demonstrated that nucleosomal complexes interfere with the accessibility of transcription and replication factors on DNA [8-9].

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Recently, Roca and coworkers demonstrated that DNA topoisomerase II relaxes nucleosomal DNA much faster than DNA topoisomerase I by analyzing the relaxing activities on different nucleosomal substrates suggesting that, in vivo, DNA topoisomerase I more than DNA topoisomerase II, could be impeded by nucleosomal structures in cleaving and rejoining DNA [10]. Actually the DNA topoisomerase I cleavage reaction on *in vivo* sequences has not been yet studied on substrates containing or not nucleosomes. In order to answer this question we evaluated the DNA topoisomerase I cleavage activity on the natural $(TTA)_{35}$ repeated sequence, both *in vitro* and *in vivo*. This sequence contains structural features presumably favouring both the DNA topoisomerase I site-specific cleavage activity and nucleosome assembly. In fact, the TTA sequence has been shown to be intrinsically flexible [11] and the TA step is locally bent [12]. These two features (i.e. flexibility and local bending) and the previous observation that DNA topoisomerase I *in vitro* cleaves efficiently the bent TA step [13] rendered the $(TTA)_{35}$ repeat a good substrate by which to evaluate the capability of DNA topoisomerase I to cleave a specific sequence, possibly in the presence of nucleosomes. In addition, using a strain [14] where the H4 synthesis is under the GAL1 promoter, we were able to study the DNA topoisomerase I activity on a sequence where the nucleosome organization is lost or maintained in glucose or galactose respectively. This approach allowed us to study, *in vivo*, the DNA topoisomerase I reaction on different chromatin arrangements.

EXPERIMENTAL

Strains, plasmids and culture media

Strains: **W303-1a** (*Mat***a**, *ade 2-1, ura 3-1, his 3-11,15, trp1-1, leu 2-3,112, can1-100*); **AMR51** (*Mat***a**, *ade 2-1, ura 3-1, his 3-11,15, trp1-1, leu 2-3,112, can1-100, TOP1::leu2*) kindly provided by R. Sternglanz; **UKY403** (*Mat***a**, *ade 2-101, his -Δ200, leu 2-3,112, lys2-801, ura3-52, trp1-Δ419,Δhhf1::his3, Δhhf2::leu2/pMH3110 (TRP1, CEN3, ARS1, UAS gal-HHF2)* kindly provided by M.Grunstein.

Plasmid pFDFG1a: The sequence –422 to +612 (from *PRE8* ATG) encompassing the $(TTA)_{35}$ region, was PCR amplified and cloned in the EcoRI site of the yeast vector pADH426 [15] yielding the plasmid pFDFG1a.

Culture media: cells were grown in minimal YNB [16], supplemented with 2% glucose or 2% galactose when appropriate.

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Enzymes and chemicals.

Restriction enzymes, T4 polynucleotide kinase and Micrococcal nuclease were from Roche. Vent(exo$^-$) polymerase from New England Biolabs; Zymolyase 100T from Seikagaku, Tokyo, Japan; CPT from Sigma; radiochemicals from Amersham.

Chromatin analysis

Micrococcal Nuclease treatment was performed as previously described [17]. MNase (2 and 4 units) was added to different aliquots of spheroplasts preparation and the samples were incubated at 25°C for 20 minutes. The DNA was then deproteinized and ethanol precipitated. RNase treatment was also performed.

Low resolution analysis

Indirect end labeling analysis [18] was performed as follows: after treatment with the appropriate restriction enzymes, the samples were electrophoresized in 1.2% agarose gels (1.75 volts/cm), transferred onto nitrocellulose membrane, hybridized to the TTA probe and detected by autoradiography.

***In vivo* induction of CPT dependent DNA topoisomerase I cleavage sites**

Cells were treated for cleavage sites induction as previously [7]. Three different aliquots were incubated with 50-100-200 μM CPT for 2 minutes at R.T. The reaction was stopped with 1% SDS, 5 mM EDTA. The DNA was then deproteinized and ethanol precipitated. RNase treatment was also performed.

Oligonucleotide primers and probes

Oligonucleotides sequences used in the extension reactions:

VEC: 5'-GGTATCGATAAGCTTGATATCG-3'

r3: 5'-CGCGTTTCCGTATTTTCCGC-3'

The oligonucleotide VEC lies at position -449 from *PRE8* ATG of the cloned sequence containing the $(TTA)_{35}$ in pFDFG1a. The r3 oligonucleotide used lies at position -267/-248 bp from the 35S 35S RNA RIS. 5'-end labelling using (γ-^{32}P)ATP and T4 polynucleotide- kinase was performed according to [19].

The probe TTA, annealing at positions +436/+232 bp, from the ATG of *PRE8* gene, was labelled by random priming.

Multiple-round primer extension and high resolution analysis of *in vivo* MNase and DNA topoisomerase I cleavage sites.

1/2 μg of genomic DNA were reacted with Vent polymerase and end-labelled oligonucleotide as described [20]. The samples were cycled 25 times through the following steps: 95°C for 5 min., 58°C for 10 min., and 76°C for 3 min. (conditions for oligonucleotide VEC). The extension

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products were phenol extracted, ethanol precipitated, dissolved in formamide buffer and analysed by 6% denaturing polyacrylamide gel electrophoresis.

***In vitro* DNA topoisomerase I cleavage sites.**

The reactions were performed as described [21] reacting 1, 5 and 10 Units of purified DNA topoisomerase I (calf thymus) with 1-2 ng of end-labeled DNA. The reactions were carried out in 10 mM Tris-HCl, pH8.0, 10 mMNaCl, 1mM MgCl2 and 100 μM CPT for 15 min at 37°C. The DNA substrate for cleavage reaction was EcoRI excised from pFDFG1a. After restriction with XhoI/XbaI the fragment was 3'-labeled, purified and further digested with SmaI or HindIII in order to uniquely orient the labelling.

RESULTS

We selected the trinucleotide repeat $(TTA)_{35}$ lying in the intergenic region of the chromosome XIII of *S. cerevisiae*, between the *RPM2* and *PRE8* genes. This sequence, as reported above, contains the structural elements presumably suitable to be susceptible to DNA topoisomerase I site-specific activity and to assemble nucleosomes.

***In vitro* high resolution analysis of DNA topoisomerase I cleavage sites on $(TTA)_{35}$ repeat.**

The DNA tract in the chromosome XIII containing the sequence $(TTA)_{35}$, was amplified by PCR with two oligonucleotides annealing at positions -474 and +755 relative to the *PRE8* ATG. The resulting fragment was cloned into the yeast vector pADH426, yielding the pFDFG1a plasmid. The XhoI/XbaI fragment from pFDFG1a was 3'-labeled, purified and further digested with SmaI in order to uniquely orient the labelling and to study the DNA topoisomerase I cleavages with a nucleotide level of resolution. The fragment was reacted *in vitro* with increasing amounts of calf thymus DNA topoisomerase I (triangle in Figure 1) in the presence of Camptothecin (CPT); this drug inhibits the rejoining reaction of DNA topoisomerase I thus creating, in the presence of a denaturing agent, single strand breaks and behaving like a single strand nuclease. As reported in Figure 1, DNA topoisomerase I strongly recognizes the TTA repeats (arrows). The regularly repeated sequence is highly cleaved (from -312 to -216) and TTA repetitions in the flanking regions are also efficiently cleaved. Asterisks indicate cleavage sites not occurring on a TTA sequence.

***In vivo* analysis of DNA topoisomerase I cleavage sites on the $(TTA)_{35}$ repeat.**

Given the results reported in Figure 1 we evaluated the DNA topoisomerase I cleavage activity on the same DNA sequence *in vivo*. WT and AMR51 (*Δtop1*) cells, transformed with pFDFG1a, were analyzed for cleavage sites induction as previously reported [7, 20]. Briefly: spheroplasts from WT

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and *Δtop1* cells were permeabilized with nystatin [17] and treated with different amounts of CPT (triangles in Figure 2). DNA was then purified and primer extended from the labelled oligonucleotide VEC (annealing on the vector sequence at -449 bp from *PRE8* ATG contained in the fragment cloned in pADH426). Only a weak cleavage profile is observed in the WT strain (Figure 2). The only detectable sites localize at the flanking regions of the triplet (arrows). In the *Δtop1* strain no cleavage sites are observed. Taken together, these data suggest that DNA topoisomerase I poorly reacts *in vivo* with the $(TTA)_{35}$ repeat despite its *in vitro* high reactivity towards the same sequence (Figure 1).

$(TTA)_{35}$ repeat is engaged in a positioned nucleosome *in vivo*.

Results reported in Fig 2 indicate that DNA topoisomerase I does not efficiently react with the $(TTA)_{35}$ tract *in vivo*. Could this low cleavage activity on the repeated sequence be due to its chromatin organization? In order to clarify this point we studied the nucleosome positioning on this DNA sequence by *in vivo* analysis with Micrococcal Nuclease (MNase).

Spheroplasts from WT cells, transformed with pFDFG1a, were treated with nystatin, digested with different amounts of MNase (triangles in Figure 3) and DNA was purified. Results are shown in Figure 3 Panel A: the *in vivo* treated samples (chromatin) were compared with those digested *in vitro* (naked DNA) to provide the susceptibility of deproteinized DNA to MNase. This comparison shows protected regions representing nucleosomal particles based on their dimension (140-160 bp) (ellipses in the figure). Nucleosome reported as #1 occupies positions from –160 bp to – 330bp in the chromatin samples, while in the naked DNA the same region appears strongly digested. This region encompasses the $(TTA)_{35}$ region (-214 to –318).

To increase the mapping resolution, we primer-extended the DNA of different aliquots from the same samples with the labeled oligonucleotide VEC. The high resolution data reported in Panel B show that the $(TTA)_{35}$ repeat is almost completely protected by the nucleosome #1 (from –349/-324bp to –189bp on multiple positions, filled arrows in Figure3 Panel B) as shown by comparing the chromatin samples with the naked DNA. In addition MNase hypersensitive sites (empty arrows) localize at the flanking regions of the mapped nucleosome.

Taken together the data reported in Figure 3 demonstrate that the $(TTA)_{35}$ repeat is actually organized in a family of positioned nucleosomes covering multiple positions, all sharing the TTA repeat in the middle of the particle.

***In vivo* chromatin organization of the $(TTA)_{35}$ sequence after nucleosome removal.**

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Could the positioned nucleosome on $(TTA)_{35}$ be responsible for DNA topoisomerase I failure in generating efficient cleavage sites *in vivo*? In order to verify this hypothesis we analyzed the *in vivo* DNA topoisomerase I cleavage activity in a yeast strain lacking both H4 genes and carrying one copy of H4 histone under the GAL1 promoter [14]. This implies that in the presence of glucose as carbon source, the chromatin organization will be lost and DNA should become accessible *in vivo* both to MNase and DNA topoisomerase I.

Yeast UKY403 cells, transformed with pFDFG1a, grown in galactose, were splitted in two aliquots, washed and resuspended in a growth medium containing galactose or glucose. After 4 hours of growth, spheroplasts were treated with different amounts of MNase. The digestion profiles shown in Figure 4, Panel A, (bulk DNA), indicate that in the GAL grown cells the nucleosomal ladder is obtained, while in the GLU cultures, a smear appears in the corresponding region of the gel. This suggests that MNase freely accesses DNA when the nucleosomal barriers are absent.

When the samples from GAL or GLU cultures were analyzed by end-labeling technique [18] as in Figure3 Panel A, a specific difference is observed (Figure 4. Panel B): GAL samples show protection of the TTA region towards MNase (nucleosome #1), as reported in Figure 3; conversely, GLU samples show a diffuse accessibility to MNase on the $(TTA)_{35}$ sequence similar to that of the naked DNA.

DNA topoisomerase I *in vivo* cleaves the $(TTA)_{35}$ sequence after nucleosomes removal.

The data reported in Figure 4, Panel B, demonstrate that the chromatin organization of the $(TTA)_{35}$ repeat is completely lost when cells were grown in glucose. Since in these conditions MNase accesses $(TTA)_{35}$ repeat with the same efficiency *in vivo* end *in vitro*, we wanted to evaluate whether also DNA topoisomerase I could reach this sequence when chromatin is not organized any more. We then analyzed the same cells for DNA topoisomerase I cleavage sites induction at high resolution (Figure 5 Panel, A). The samples were primer extended starting from the labeled oligonucleotide VEC: the reactivity of DNA topoisomerase I in the GLU culture is highly enhanced compared to that observed in the GAL culture. In addition, the cleavage profile of GLU samples (arrows) almost completely overlaps to that obtained *in vitro* (see Figure 1). Then we analyzed the same samples in a different chromosomal location (the NTS2 region of the rDNA) as a control. In fact, we have previously shown [7, 20] that in this nucleosome free region [22] a strong DNA topoisomerase I cleavage site occurs at -171 bp from R.I.S [7]. Results are shown in Figure 5, Panel B. When the and the oligonucleotide r3 [-268bp from the 35S RNA start site (R.I.S.)] was primer extended both conditions (GLU/GAL, i.e. nucleosomes presence/absence respectively) show the -171 cleavage site, demonstrating the same reactivity of DNA topoisomerase I in a nucleosome free

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region when H4 transcription is on or off (Figure 5, Panel B). Taken together the data reported in Figure 5, Panels A and B, demonstrate that nucleosomes represent a barrier for localized DNA topoisomerase I activity.

In order to exclude that the observed differences in accessibility to TTA region are due to a different amounts of DNA topoisomerase I in the two growth conditions, we measured both TOP1 mRNA production and DNA relaxing activity (not shown). All evaluations showed a slight increase in mRNA and DNA topoisomerase I activity in cells from GLU cultures; however such a small difference does not justify the strong enhancements observed in the in vivo cleavage profile (Figure 5 PanelA).

DISCUSSION

It is generally accepted that all sequences in DNA can be substrates for DNA topoisomerase I relaxing activity, even though DNA topoisomerase I seems to have a higher reactivity towards some particular sequences showing structural features like DNA bending [13, 23]. The high resolution mapping of *in vivo* DNA topoisomerase I site specific activity concerns nucleosome free regions [5-7]. In order to verify whether nucleosomes affect DNA topoisomerase I site-specific cleavages, we studied a natural DNA sequence repeating 35 times the TTA trinucleotide. Such a sequence provides: the locally bent TA step [12], presumably a good substrate for local DNA topoisomerase I cleavage induction and an intrinsic flexibility [11] potentially useful to efficiently assemble nucleosome.

The TTA trinucleotide repeat has proved to be efficiently cleaved *in vitro* by DNA topoisomerase I (Figure 1) in the repeated tract and also in its surrounding regions, confirming the preference of the enzyme for this sequence. Conversely, an *in vivo* approach showed a reduced reactivity of the same sequence towards DNA topoisomerase I. Thus, it is conceivable to hypothesize that chromatin structure affects DNA topoisomerase I cleavages. Recently Roca and coworkers [10] observed that DNA topoisomerase II is more efficient than DNA topoisomerase I in releasing topological stress from nucleosomal substrates; this supports the hypothesis that nucleosomes may represent a barrier for DNA topoisomerase I activity. At this purpose a distinction between the global relaxing activity and the local site specific cleavage reaction, should be taken under consideration. In fact a different chromatin organization in a given substrate, could be not determinant in the whole relaxing activity of DNA topoisomerase I: indeed the enzyme can release torsional stress acting on different sites in the substrate. Conversely, when a given sequence is analyzed in terms of cleavage activity exerted by DNA topoisomerase I, the absence/presence of a nucleosome could be very relevant and this latter point was investigated by our experimental system.

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In order to verify this hypothesis, we studied the *in vivo* chromatin organization of the $(TTA)_{35}$ tract. As reported in Figure 3, a positioned nucleosome occupies the $(TTA)_{35}$ region possibly hindering the DNA topoisomerase I cleavage activity. Then we employed a yeast strain carrying a plasmid in which the H4 gene is under the GAL1 promoter. This allowed us to switch on/off the synthesis of this histone, depending on the carbon source. In the GAL condition the presence of nucleosomes impairs DNA topoisomerase I approach to the $(TTA)_{35}$ sequence. Conversely, in the GLU condition, when H4 production is repressed, no organized chromatin is observed on the TTA repeat and DNA topoisomerase I increases its sequence specific activity, particularly on the $(TTA)_{35}$ tract (Figure 4).

In order to evaluate the cleavage difference of DNA topoisomerase I in vivo, in vitro and when chromatin is destructured (H4 no more synthesized in Glucose medium), we quantified the DNA topoisomerase I digestion profiles and the data are compared in Figure 6. As it appears, when chromatin is regularly organized (in vivo and in vivo GAL, see also chromatin profiles in Figs.3 and 4) about 5-6% of the digested material is represented by the TTA repeat. Conversely the relative digestion of the TTA repeat when samples are reacted in vitro with DNA topoisomerase I reaches values near to 30%. Also the dissolution of regularly organized chromatin allows DNA topoisomerase I to digest the TTA repeat in vivo, but with an efficiency of 15% (about three times higher than on the regular chromatin).

Thus we can conclude that: i) DNA topoisomerase I efficiently reacts with the TTA repeat; ii) the $(TTA)_{35}$ sequence, the longest and most stable among the simple repeated sequences in *S. cerevisiae* [24], is organized in a positioned nucleosome and possibly this can account for its high stability: in fact each nucleosome stores one negative supercoil [25-26], thus preventing DNA denaturation and induction of conformational alterations responsible for genetic instability [27]; iii) the positioned nucleosome on the $(TTA)_{35}$ sequence represents a hindrance to the DNA topoisomerase I activity. This last conclusion, based on the GLU/GAL experiments, represents the first formal evidence that DNA topoisomerase I cannot react with nucleosomal DNA.

Despite the well known chemistry concerning the DNA topoisomerase I mechanism of action and the interference with small inhibitors largely employed in chemotherapy, so far no details are known about the possible interference that nucleosome could represent *in vivo* towards topoisomerization reaction. Here we provide direct evidences clarifying this task.

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FIGURE LEGENDS

Figure 1. *In vitro* DNA topoisomerase I cleavage site on TTA sequence. 1, 5 and 10 U of calf thymus DNA topoisomerase I (triangle) were reacted with the 3' labelled TTA containing DNA sequence in the presence of Camptothecin (CPT). Mapping of the fragments, separated by sequencing gel, was obtained comparing the migration of samples with the size marker (M) and sequencing lane of A (A). Black arrows and asterisks indicate *in vitro* DNA topoisomerase I cleavages on TTA or different sequences respectively. A schematic map containing the genes flanking the TTA repeated sequence is reported at the right side. Numbering refers to PRE8 ATG.

Figure 2. *In vivo* DNA topoisomerase I cleavage site on TTA sequence
Spheroplasts from WT or *top1Δ* cells were treated with 0 (U), 50, 100 and 200 µM CPT (triangles). The fragments resulting from DNA topoisomerase I activity were primer extended from oligonucleotide VEC (white arrow) annealing at position -499 from *PRE8* ATG. Black arrows indicate DNA topoisomerase I cleavage sites. M: pBR322/MspI, size marker. A: sequencing lane. At the right part of the figure a representation of the analyzed region is shown.

Figure 3. Nucleosome positioning on the TTA sequence
Panel A. Nystatin-permeabilized spheroplasts were treated with 0, 2, and 4 U respectively of micrococcal nuclease (MNase). Purified DNA was restricted with BamHI and run in 1.2% agarose gel, transferred onto a nitrocellulose membrane and hybridised with the TTA probe. **Chromatin**: *in vivo* digested samples; **Naked**: *in vitro* digested samples of deproteinized DNA; U: untreated DNA; M1: size marker (1kb ladder); M2: size marker (123bp ladder). Black arrows indicate MNase cleavage sites.
Panel B: DNA was digested *in vivo* (Chromatin) and *in vitro* (Naked DNA), after deproteinization, with 0-1-2-4-8 U of MNase and primer extended with the labelled oligonucleotide VEC (position -449). Lane M size marker (pBR322/MspI); A: sequencing lane; U: untreated DNA. Filled and empty arrowheads indicate protections and enhancements respectively; map on the right shows nucleosome with multiple positions (ellipses). All numbering refers to *PRE8* ATG.

Figure 4. Nucleosome organization on TTA sequence upon nucleosomes removal.
Panel A: Spheroplasts from cells grown in galactose (GAL) (transcribing H4 gene) or in glucose (GLU) (non-transcribing H4 gene) were subjected to MNase digestion (as in Figure 3). DNA was

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purified and analyzed by agarose gel electrophoresis. Lane M: size marker (1kb ladder); U: untreated DNA. Arrows show cleavages of MNase on DNA.

Panel B: Spheroplasts from cells grown in galactose (GAL) (transcribing H4 gene) or in glucose (GLU) (non-transcribing H4 gene) were subjected to MNase digestion as reported in Figure 3 and compared with MNase digestions on deproteinized DNA (Naked). Lane M: size marker (1kb ladder); U: untreated DNA; ellipses indicate nucleosomes.

Figure 5. DNA topoisomerase I cleavage activity on TTA sequence upon nucleosomes removal

Panel A: Spheroplasts from cells grown in galactose (GAL) (transcribing H4 gene) or in glucose (GLU) (non-transcribing H4 gene) were subjected to DNA topoisomerase I cleavage sites in the presence of camptothecin; induction as in Figure 2. Lane M: size marker (pBR322/MspI); U: DNA from untreated cells. Arrows indicate DNA topoisomerase I cleavage sites. Numbering refers to *PRE8* ATG. At the right part of the figure a schematic representation of the analyzed region is shown.

Panel B: Samples as in Panel A were primer extended from oligonucleotide r3 in order to show DNA topoisomerase I cleavage sites on NTS2 region of ribosomal genes. C, T: sequencing lanes. U: untreated sample; M: size marker.

Figure 6. DNA topoisomerase I accessibility on different contexts. Different DNA topoisomerase I digestion profiles from: in vitro (white bar), in vivo (dashed bar), in vivo GLU (non-transcribing H4 gene) (black bar) and in vivo GAL (transcribing H4 gene) (dotted bar) where evaluated by densitometric scanning. Data represent the % of cleavage activity on the TTA repeat.

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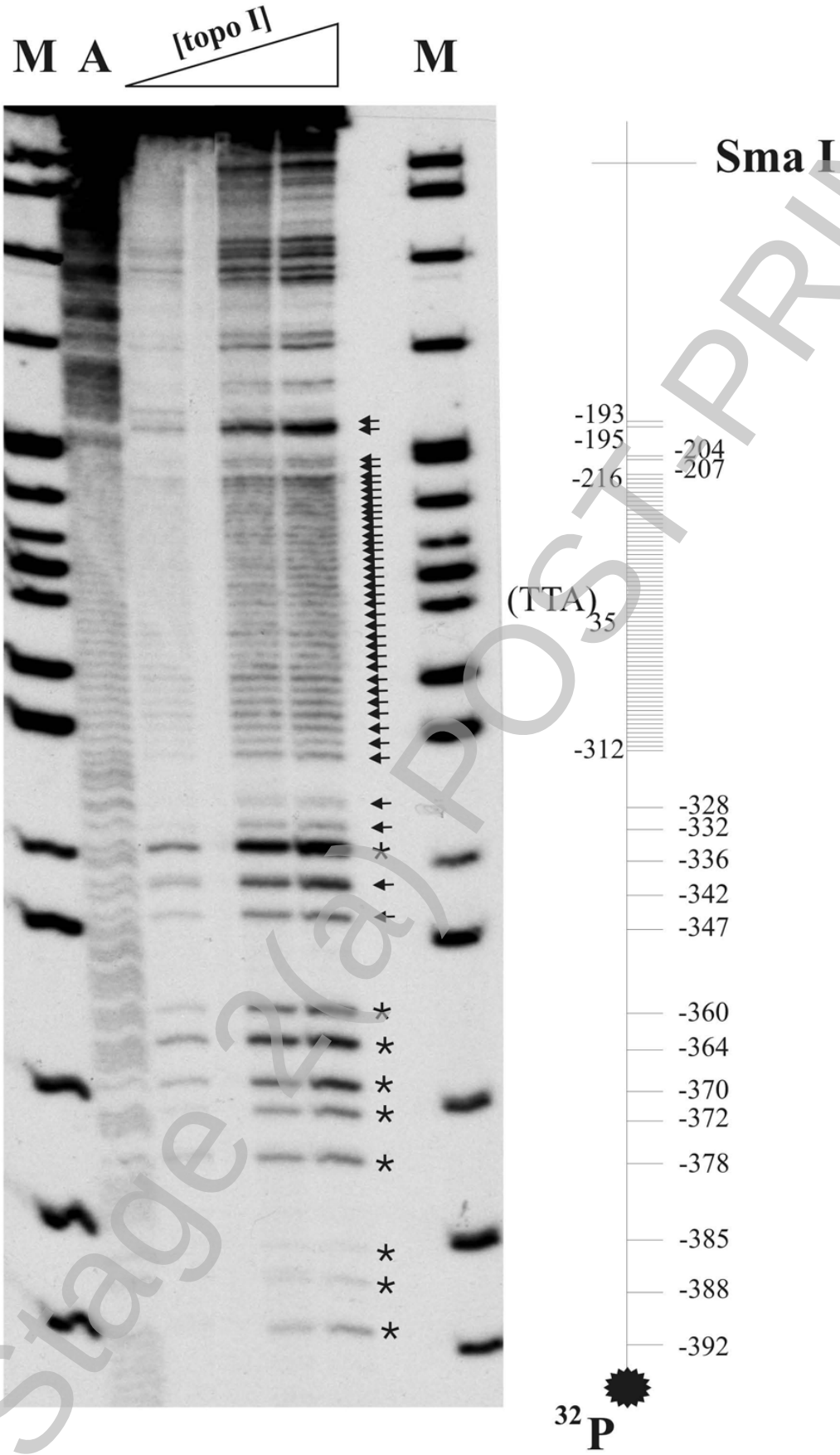

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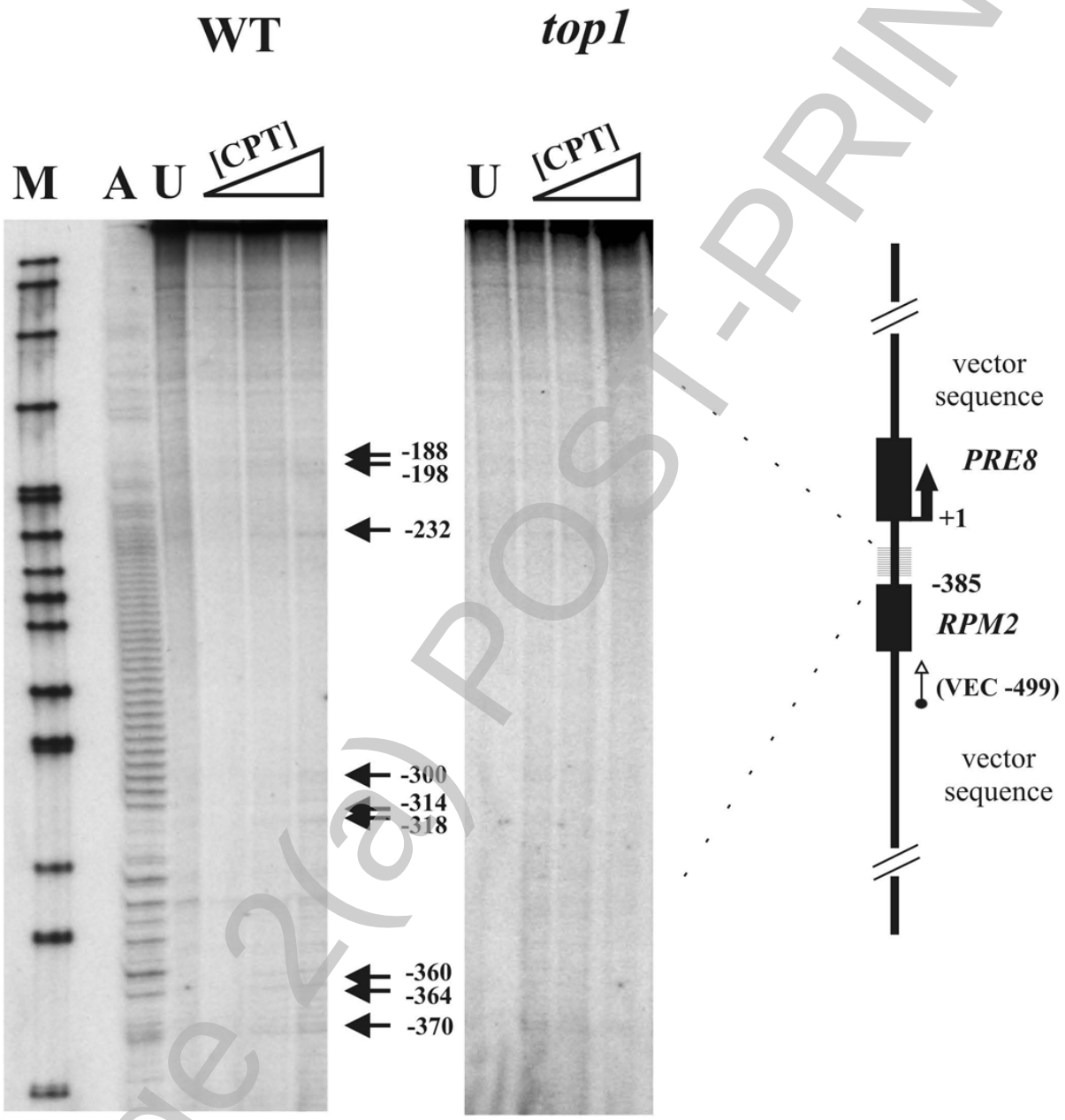

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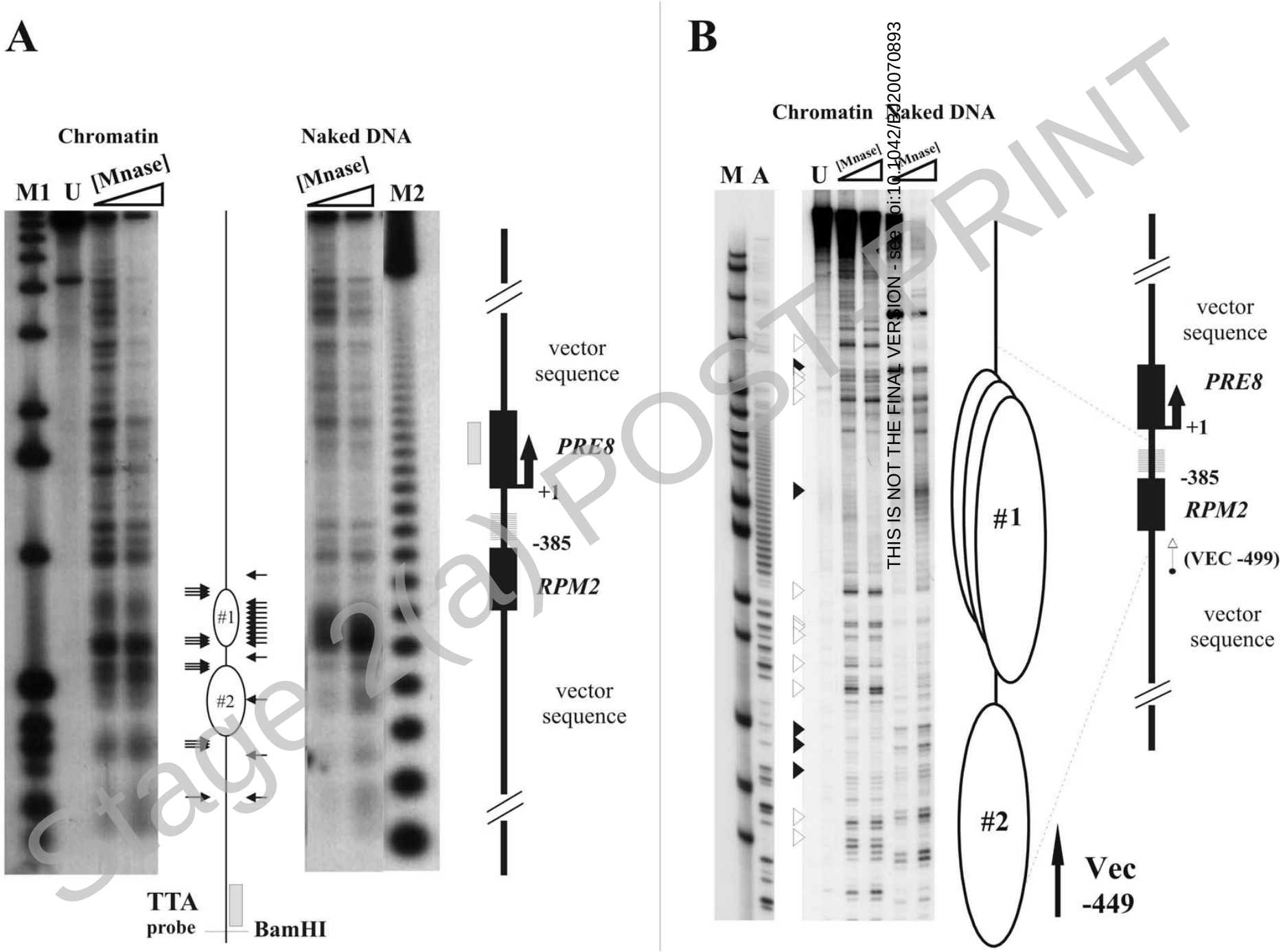

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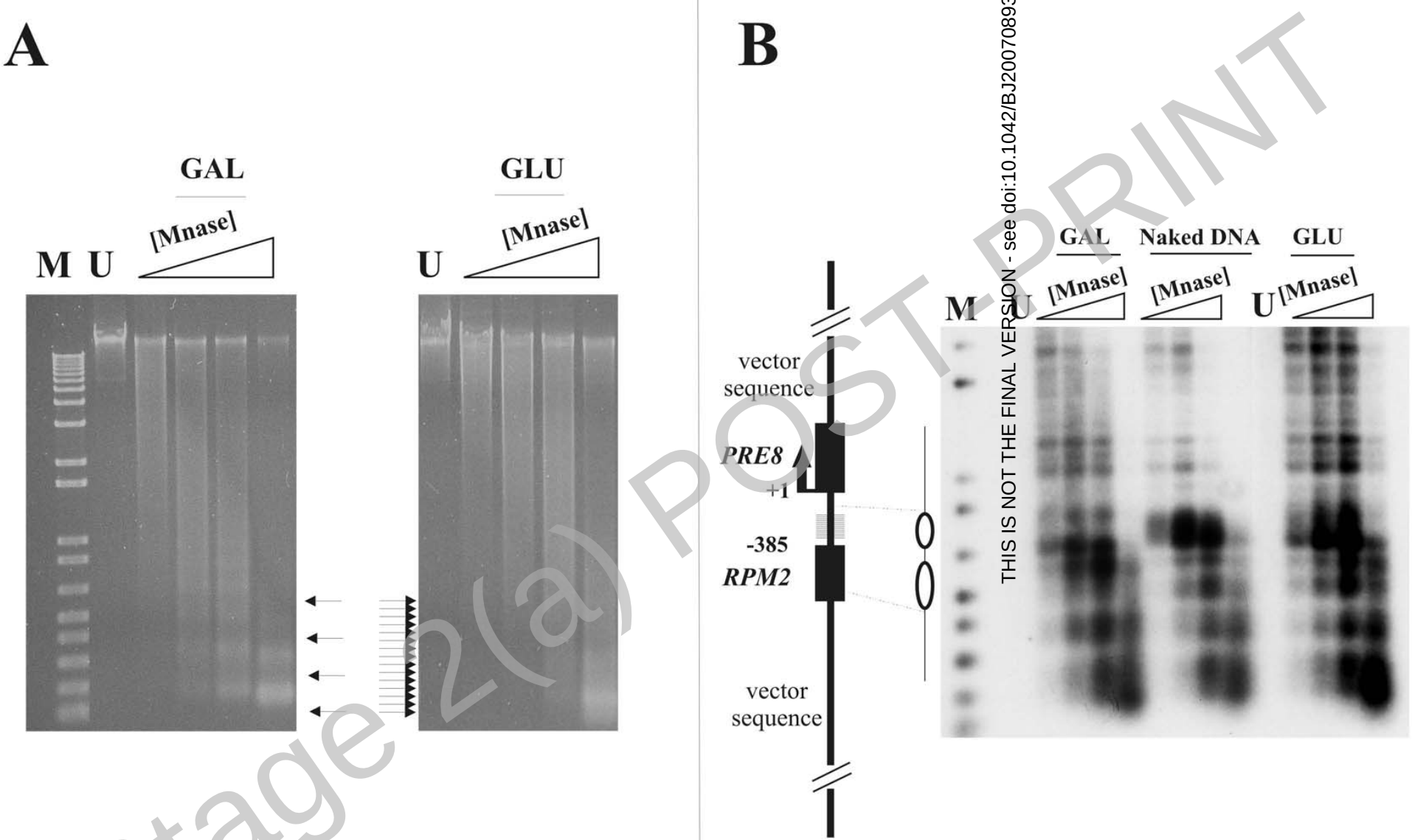

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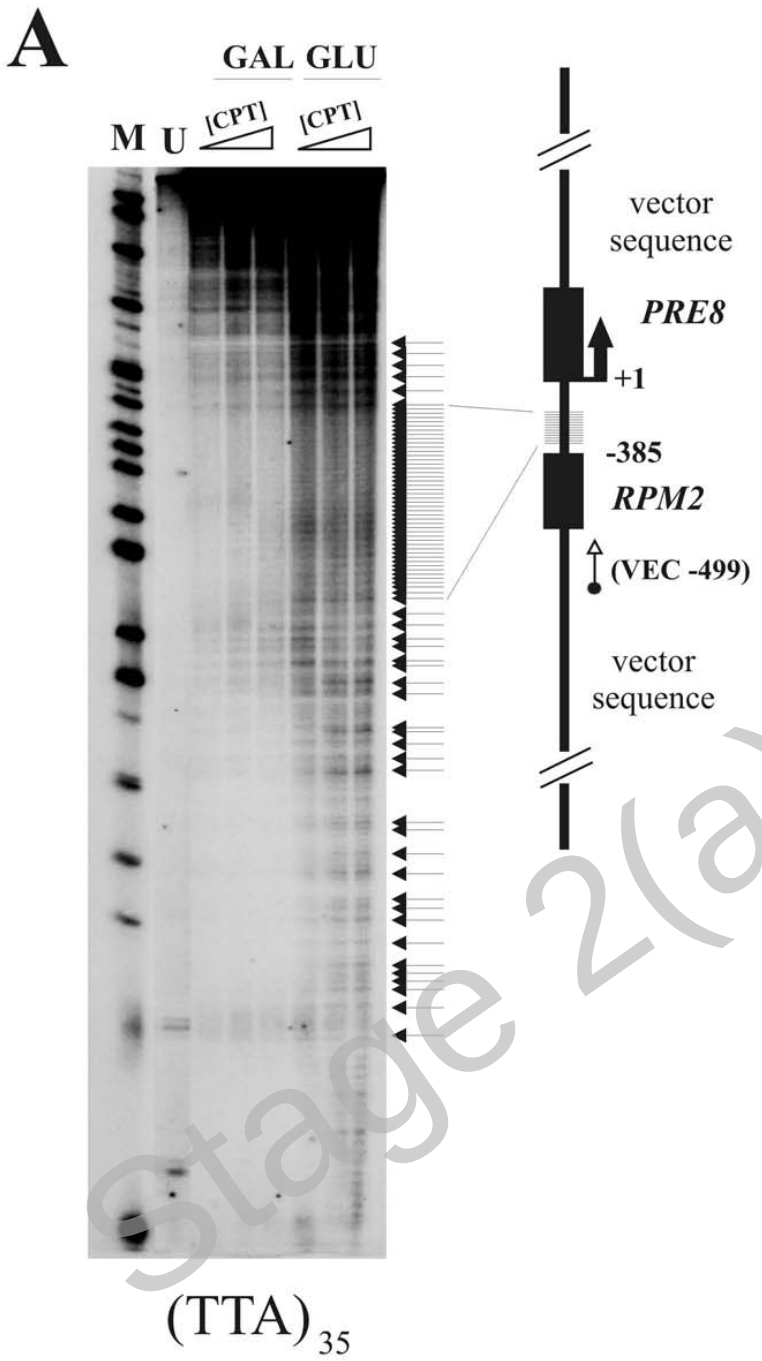

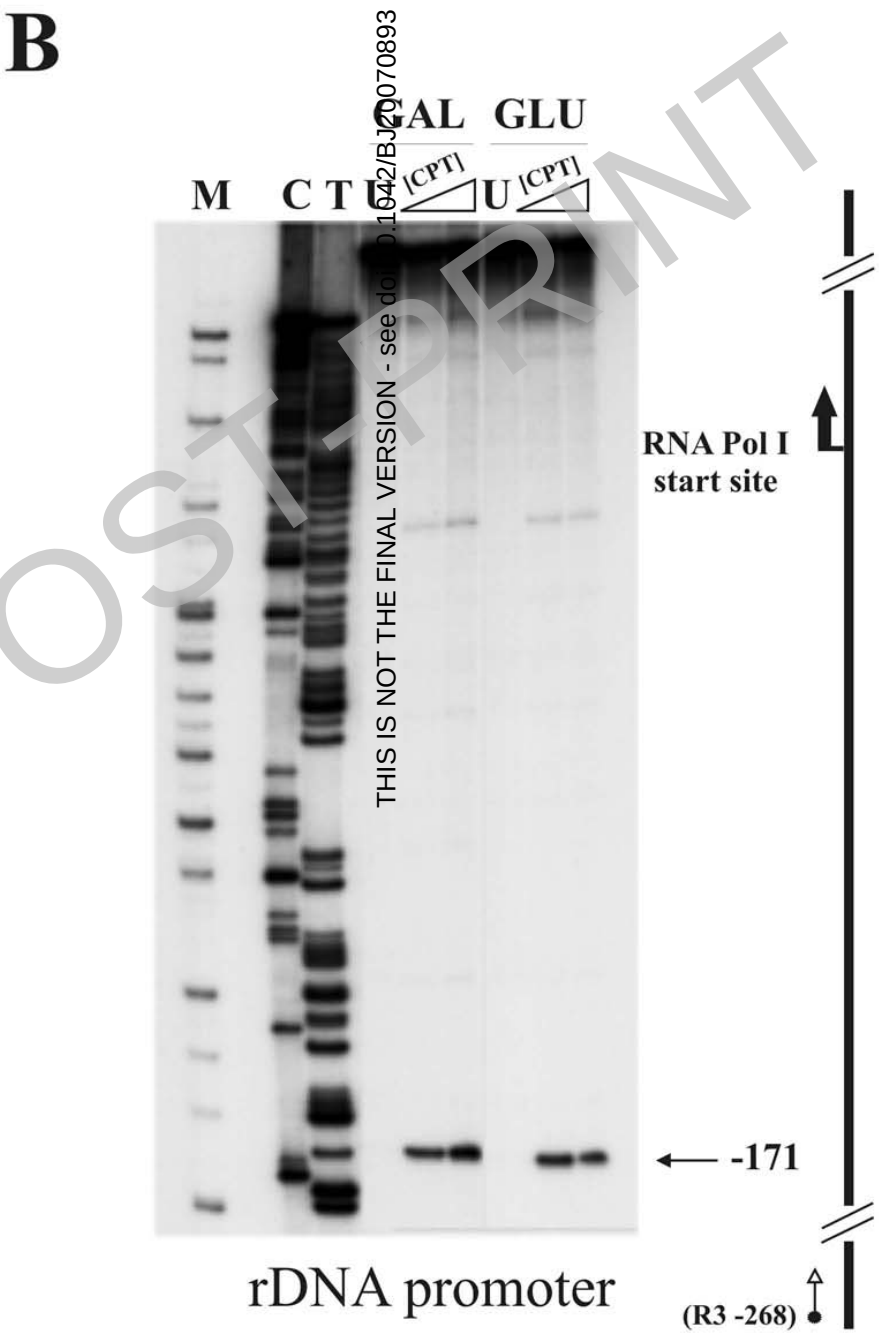

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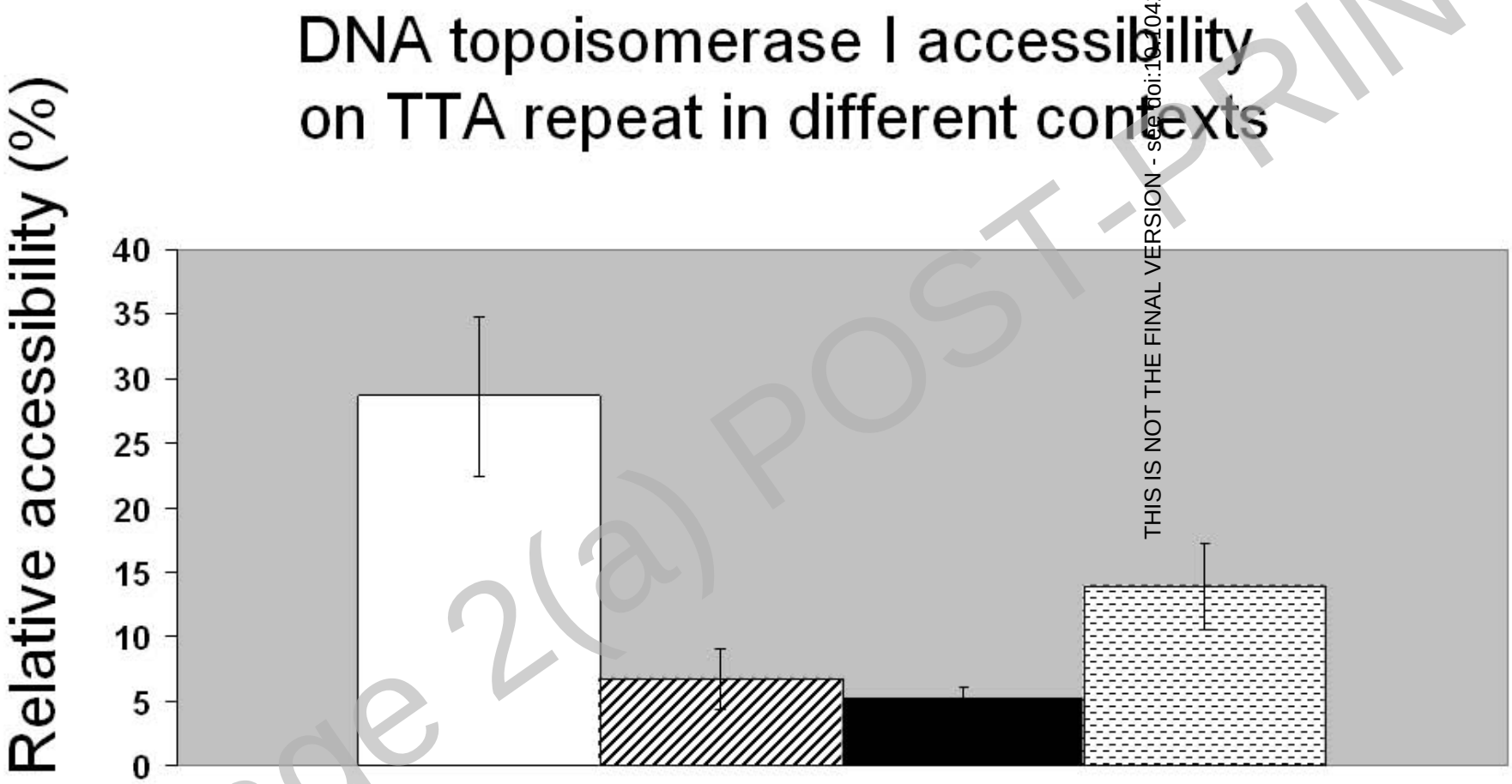

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