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Structure of RNA polymerase II promoters. Conformational alterations and template properties of circularized *Saccharomyces cerevisiae GAL1–GAL10* divergent promoters

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A DNA fragment encompassing the *Saccharomyces cerevisiae GAL1–GAL10* divergent promoters (914 bp) has been circularized *in vitro* with T4 DNA ligase. We have defined a set of conditions that allows the production of a series of nine topoisomers covering a range from relaxed to highly negatively supercoiled DNA. Topoisomers were recovered in pure form from agarose gels and were analysed singly for the presence of sites sensitive to the single strand-specific endonuclease P1. In this way, the occurrence of conformational alterations as a function of the linking deficiency of the closed DNA domain has been determined. Interestingly, sites of P1 hypersensitivity localize on the three sequences identified as relevant for the *in vitro* transcription of the *GAL1* moiety of the divergent promoter: the upstream activator sequence (UAS), the TATA sequence, and the RNA initiation site (RIS). *In vitro* transcription with purified *S. cerevisiae* RNA polymerase II shows that activation of transcription parallels the appearance of conformational alterations on the UAS, the TATA and the RIS sequences.

Key words: Saccharomyces cerevisiae/GAL1–GAL10 promoters/ RNA polymerase II/conformation

Introduction

Evidence that the 'conformational information' (Rich, 1983) contained in the secondary structure of DNA in promoter regions is involved in the regulatory process of transcription has been provided (Wells *et al.*, 1980; Smith, 1981; Elgin, 1984). Alteration of DNA structure may result from covalent modification, interaction with proteins and/or torsional stress. We describe here the effects of torsional stress on a complete eukaryotic promoter region, considering that the resulting conformational alterations may reflect the topological organization of promoters and considering also that small topological modifications are induced by regulatory proteins (Reynolds and Gottesfeld, 1983; Wu and Crothers, 1984; Porschke *et al.*, 1984).

The existence of sites that are sensitive to S1, P1 or other single strand-specific endonucleases in 5′ regions of eukaryotic genes in supercoiled DNA and in chromatin has been reported (Larsen and Weintraub, 1984; Weintraub, 1983; Selleck *et al.*, 1984; Kowalski, 1984). DNase I and S1 nuclease hypersensitivity mapping to the 5′-flanking region of eukaryotic genes in chromatin domains with transcriptional activity reflect altered DNA structure (Elgin, 1984, and references therein). In some instances sensitive sites correspond to special sequences (homopurine–homopyrimidine stretches, Mace *et al.*, 1983; Nickol and Felsenfeld, 1983; Schon *et al.*, 1983) which, under torsional stress, are present in an altered double-stranded conformation (Schon *et al.*, 1983; Evans *et al.*, 1984; Cantor and Efstratiadis, 1984; Selleck *et al.*, 1984).

In topologically closed domains torsional stress is a global property. It is conceivable that part of the free energy of supercoiling induces and stabilizes structural heterogeneity in the promoter region, thus allowing or facilitating interaction with RNA polymerase and/or with regulatory proteins.

The relationship among superhelicity, modification of the secondary structure of DNA and gene regulation has been outlined (Wells *et al.*, 1980; Smith, 1981; Elgin, 1984; Lilley, 1983, and reference therein) and DNA supercoiling is considered relevant to the regulation of gene expression (Smith, 1981; Harland *et al.*, 1983; Abraham *et al.*, 1983, and references therein). Chromosomes are organized in negatively supercoiled domains (Benyajati and Worcel, 1976; Laemmli *et al.*, 1978) and, although it is not yet known to what extent chromosomal DNA is under actual (not constrained by protein binding) torsional stress, it is clear that in both prokaryotes (Pruss *et al.*, 1982; Di Nardo *et al.*, 1982) and eukaryotes (Abraham *et al.*, 1983) superhelical density is regulated. Genetic and biochemical evidence (Smith, 1981; Gellert, 1981) show that certain bacterial promoters are active *in vivo* only when the DNA is fully supercoiled. In eukaryotic systems, the influence of the topological state of the DNA on *in vivo* (Abraham *et al.*, 1983), *in vitro* (Lescure *et al.*, 1983; Ballario *et al.*, 1981; Carnevali *et al.*, 1982, 1983, 1984; Di Mauro *et al.*, 1985) or *in ovo* (Ackrigg and Cook, 1980; Pruitt and Reeder, 1982) transcription has been shown.

The model system that we have analysed consists of a covalently closed domain containing only the intergenic region of the divergently transcribed *Saccharomyces cerevisiae GAL1–GAL10* genes (914 bp). RNA polymerase II promoters of *S. cerevisiae* contain two essential elements: a sequence homologous to the TATA-box and an upstream activatory sequence (UAS) (Guarente, 1984). Yeast UAS are *cis*-acting elements that modulate transcription from upstream of the RNA initiation site. The *GAL* UAS is the target for the interaction of the *trans*-acting positive effector *GAL4* (Johnston and Hopper, 1982; Laughon and Gesteland, 1982). Yeast UAS are thought to correspond to the enhancer regions described in mammalian cells (Benoist and Chambon, 1981; Gruss *et al.*, 1981) because of their distance from the initiation region and their orientation-independent mode of action (West *et al.*, 1984). Their activatory function can be transplanted to other genes (Guarente *et al.*, 1982) and the distance between the UAS and TATA-box is not critical for function (Yocum, cited in Brent and Ptashne, 1984). UAS_G function has been localized to be a 75-bp sequence (West *et al.*, 1984) displaying two sites of dyad symmetry. It appears (Giniger *et al.*, cited in Brent and Ptashne, 1984) that one of these symmetrical elements is sufficient for UAS_G function. We also note that the sequences that form the rotational symmetries are homogeneous homopurine–homopyrimidine stretches.

In the *GAL1–GAL10* intergenic region, supercoiling-dependent P1 nuclease-sensitive sites are located at positions that have been identified as relevant for the functioning of the *GAL1* promoter.

Results

Effect of torsional stress on small-sized DNA domains

Topological characterization of covalently closed 914 bp GAL1–GAL10 domains. When eukaryotic sequences are inserted into bacterial plasmids, the topological state of the DNA under native or *in vitro*-induced torsional stress depends, collectively, upon the size and nature of the components; the juxtaposition of vector and insertions leads to a complex topological interplay that determines the observed pattern of S1 hypersensitivity and the *in vitro* template properties of the chimaeric construct (predicted by Benham, 1982, 1983 and verified by Carnevali *et al.*, 1983, 1984; Di Mauro *et al.*, 1985). Thus two conditions have to be met in order to study the effect of torsional stress on DNA sequences regulating eukaryotic transcriptional activity: first, foreign sequences, whether from the vector or from unrelated genes, must be absent; second, the regulatory region has to be studied as a unit. In topological terms, the behaviour of a DNA sequence is not expected to be the sum of the behaviours of its components (Di Mauro *et al.*, 1985; Benham, 1982).

The study of the closed domain obtained by *in vitro* circularization of the 914-bp *GAL1–GAL10* intergenic region satisfies both of these requirements.

The intergenic region of the *S. cerevisiae* genes coding for galactokinase (EC 2.7.1.6) (*GAL1*) and uridine diphosphoglucose 4-epimerase (EC 5.1.3.2) (*GAL10*) is well characterized (Guarente, 1984; Brent and Ptashne, 1984; West *et al.*, 1984; Yocum *et al.*, 1984; Johnston and Davis, 1984; Guarente *et al.*, 1982). Its sequence is known and regulatory functions have been assigned to some of its segments. We used a 914-bp segment encompassing (Guarente *et al.*, 1982; Yocum *et al.*, 1984) the whole intergenic region, the two transcription initiation sites, 94 and 143 bp of the coding sequences of *GAL1* and *GAL10* respectively. Circularization was obtained by *in vitro* ligation of the two *Eco*RI extremities with T4 ligase. The topology of the circular molecules can be modulated by ligating in the presence of different concentrations of an intercalating agent, and/or by varying the temperature at the moment of closure.

No detailed description of the topological behaviour of DNA fragments of this size, ligated under different conditions, has ever been provided. The following section provides such a description.

The basic topological property of a closed circular DNA molecule is its constant linking number. This topological entity was originally described as the sum of the number of twists that either strand forms around the central axis of the molecule and of the writhing number that measures the shape of the central axis (Vinograd *et al.*, 1968). In recent redefinition of the terminology of DNA supercoiling (Wang *et al.*, 1983) τ, the number of superhelical turns, is retained as the only readily measurable component of the writhing; superhelical density, σ, is defined as $\sigma = \alpha - \alpha^\circ/\alpha^\circ$ where α is the linking number, and α° is the linking number of the same DNA free of torsional stress, i.e. relaxed. In the same definition: $\tau \equiv \alpha - \alpha^\circ$.

Our aim was to analyze the onset of alterations (identifiable by changed susceptibility to P1 endonuclease, Kowalski, 1984) of the twisting of the DNA as a function of increasing torsional stress. Therefore we have defined a protocol for the production of a continuous series of topoisomers with increasing τ.

914-bp fragments were circularized in the presence of low concentrations of ethidium bromide (EtdBr) (small intervals — Figure 1a,b). We observed a smooth increase in average τ as a function of the concentration of the intercalating agent. A detailed analysis of the energetics of DNA twisting has been presented recently (Shore and Baldwin, 1983a, 1983b). Below a critical length (between 880 and 1000 bp) fluctuations in writhe are disfavoured. This is in agreement with Le Bret's (1979) theory for the onset of writhing that predicts that, under torsional stress, about two turns of linking are taken up in twisting before writhing begins. Whatever the mechanisms by which writhing may be facilitated (Benham, 1982; Le Bret, 1978, 1979; Vologodskii *et al.*, 1979), the initial torsional stress will be taken up by twisting fluctuations and only at higher stress will writhing occur (Le Bret, 1979; Shore and Baldwin, 1983a, 1983b).

In the 914-bp circular *GAL1–GAL10* DNA, variations of τ already begin at the lowest concentration of EtdBr tested. The stepwise decrease of the average linking number reported for small (~250 bp) DNA fragments (Shore and Baldwin, 1983a, 1983b) is not observed. Figure 1, panels c and d, reports the formation of topoisomers as a function of a wider spectrum of EtdBr concentrations. Figure 1e is an example of two-dimensional gel (2-D) electrophoresis used to unambiguously determine the sign (+ or −) of the topoisomers (left) and to increase the resolution (right) of agarose electrophoresis for the topoisomers with high τ values that could not be resolved in the first dimension. The topoisomers produced at the lower concentrations of EtdBr (Figure 1c, lanes 1–5) appear in the gel as positively supercoiled. This effect is due to the conditions of electrophoresis that reduce negative superhelicity, $\Delta\tau$, by 1 relative to the conditions of ligation, as shown in Figure 1f. This experiment describes the topological behaviour of a set of selected topoisomers when run in a 2-D gel. In the first dimension a Tris–acetate–EDTA buffer was used for both samples (left and right). In the second dimension a Tris–acetate–EDTA buffer (left) or ligase buffer (right) was used. The same topological variations have been documented both for large (Depew and Wang, 1975; Kikuchi and Asai, 1984) as well as for small-sized DNA domains (Shore and Baldwin, 1983a, 1983b). We number topoisomers according to their position and the sign which they show under standard gel conditions (Tris–acetate–EDTA buffer, 20°C). When necessary, the appropriate corrections will be specified.

From the titration reported in Figure 1, it is evident that, with one exception, the complete series of topoisomers, from T_{+2} to T_{-6}, can be produced. The highest negative superhelical density ($-\sigma$) that we obtain *in vitro* with ligase is 0.102. Under the electrophoretic conditions used, T_{-1} is absent. It appears in different gel conditions, as, for example, in the presence of EtdBr or in gels run at temperatures higher than 30°C (data not shown).

To exclude the possibility that the absence of T_{-1} might be a peculiarity of the ligase reaction, we produced a series of topoisomers using another topology-modifying system. DNA was ligated at maximal torsional stress ($T_{-6,7}$) with ligase (Figure 2a), purified through extraction from agarose gel, then relaxed with topoisomerase I. The products obtained after various times of relaxation were analyzed on a 2-D gel (Figure 2b,c,d and e). After this procedure T_{-1} is again missing. This experiment supports the interpretation that T_{-1} is not unstable in the reaction that produces it (ligase or topoisomerase I) but rather that it changes conformation once it is brought to standard conditions of electrophoresis.

Mapping of P1 endonuclease-hypersensitive sites (PHS) on different topoisomers

Localization and evaluation of the stability of alternative structures in supercoiled DNA or chromatin has primarily been obtained using single strand-specific endonucleases (Lilley, 1981; Panayotatos and Wells, 1981; Cantor and Efstratiadis, 1984; Evans *et*

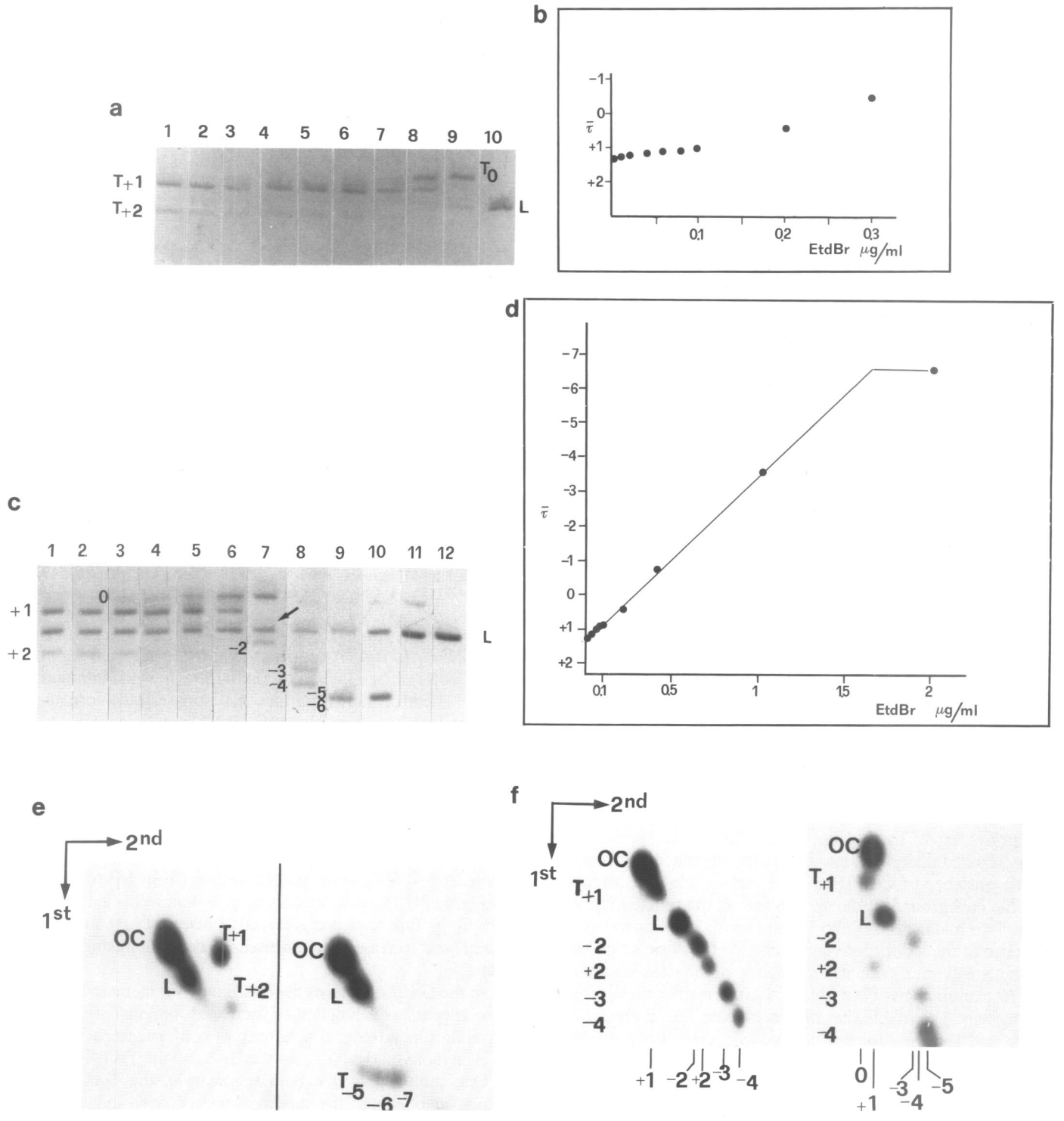

Fig. 1. Topoisomers of *GAL1–GAL10* DNA obtained by ligation in different concentrations of EtdBr. **Panel a** shows **(lanes 1–9)** the products of ligation of 1 μg of purified *GAL1–GAL10* intergenic region DNA ligated at 10°C in the presence of 0, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2 and 0.3 μg/ml EtdBr and resolved by electrophoresis in 2% agarose at 20°C. **Lane 10** is the unligated linear DNA. The sign and the number of superhelical turns (τ) of the produced topoisomers is indicated. L = linear DNA. **Panel b** is a graphical representation of the average τ determined by scanning of the data reported in **a**. **Panel c**, as in **a** shows the same DNA ligated in the presence **(lanes 1–11)** of 0, 0.01, 0.05, 0.075, 0.1, 0.2, 0.4, 1, 2, 5 and 10 μg/ml EtdBr. **Lane 12** is the unreacted linear DNA. **Lane 11** shows that high concentrations of EtdBr are inhibitory to ligation. **Panel d** is a graphical representation of the average τ determined from the data shown in **c**. The curve levels off at 1.6 μg/ml EtdBr, as observed in other titration experiments (not shown). **Panel e** shows the 2-D gel electrophoresis (2% agarose) of samples ligated in the presence of 0 (left) or 2 μg/ml (right) of EtdBr. DNA was terminally labelled with [γ-^{32}P]ATP before ligation. Electrophoresis in the second dimension is in the presence of 0.05 μg/ml EtdBr. Open circular (O.C.) and linear (L) DNA are mostly degradation products. **Panel f** shows 2-D gel electrophoresis of a mixture of selected labelled topoisomers produced as follows. Linear DNA was ligated in the presence of varying concentrations of EtdBr and isolated as single species from agarose gel; topoisomers T_{+2}, T_{+1}, T_{-2}, T_{-3} and T_{-4} were then mixed with linear and nicked circular DNA. **Left:** both first and second dimension in Tris–acetate–EDTA buffer; **right:** first dimension in Tris–acetate–EDTA, second in ligase buffer (with the omission of BSA and dithiothreitol). The vertical lettering identifies the τ in the first dimension, the horizontal in the second. The slower migration in ligase buffer is evident. The relevant point of this experimnet is that in ligase buffer, in the second dimension, migration is shifted by 1 τ toward negative.

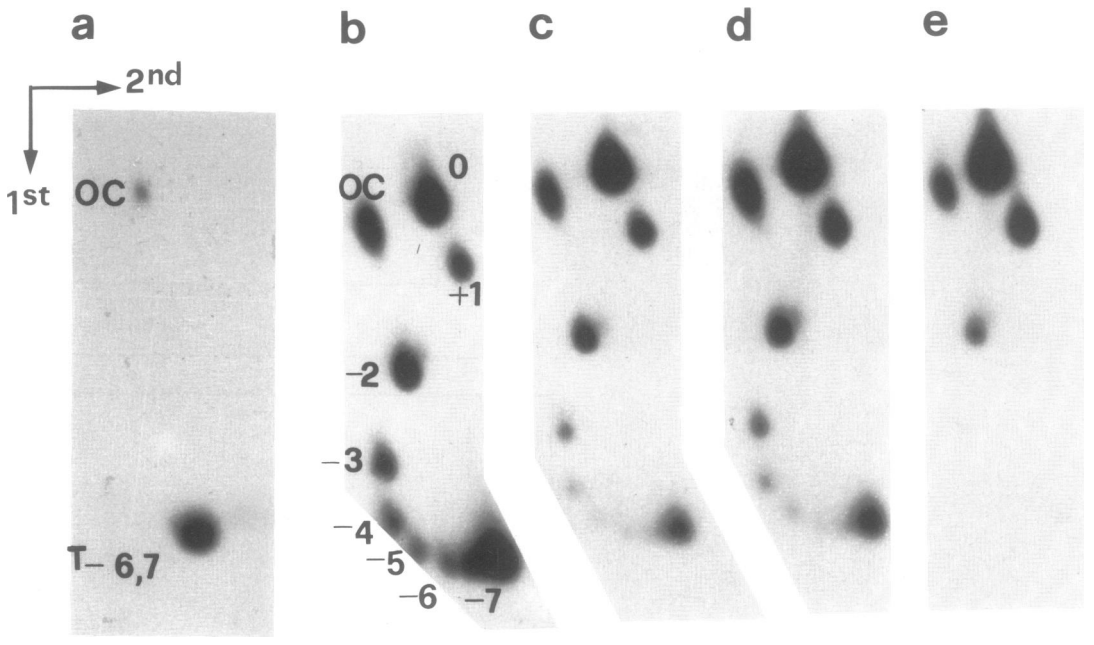

Fig. 2. Kinetics of relaxation of $T_{-6,7}$ by topoisomerase I. The terminally labelled 914-bp fragment was ligated under conditions programmed to produce T_{-6} and T_{-7} (ligation in 1.2 μg/ml, EtdBr, 2°C). The mixture of T_{-6} and T_{-7} was purified through agarose gel electrophoresis, recovered and treated with DNA topoisomerase I for 0, 0.5, 2, 4 and 20 h (**panels a–e**). The picture shows the autoradiogram of a 2-D gel run in the first dimension without EtdBr, in the second in the presence of 0.065 μg/ml EtdBr. The intercalating agent in the second dimension of electrophoresis is used to allow an unambiguous ordering and resolution of the topoisomers. Sample e is the terminal product of the relaxation reaction. The numbers indicate the sign and the number of superhelical turns.

al., 1984). This method has been criticized because nucleases might induce structures like cruciforms through their binding (Courey and Wang, 1983; Gellert *et al.*, 1983) and because — especially in the case of S1 — the acidic pH of the enzymatic digestion medium might itself cause structural alterations. Therefore P1 should be considered not an absolute, but rather a relative probe of DNA structure, and its use be limited to providing evidence of the relative differences of different samples.

Topoisomers of the 914-bp *GAL1–GAL10* intergenic region were prepared under various conditions, separated and analyzed for the presence of PHS (Figures 3 and 4). Figure 3 describes both the background of the system and an unexpected phenomenon. Two major bands (lane 1, solid arrow pointing toward the sequence at the side), deriving from two DNA sites that break spontaneously, are evident. Orientation of the sites towards the *GAL10* extremity has been obtained using restriction with *Hha*I (not shown). The fragile sites map at 147 and 156, corresponding to one end and the center of a dyad symmetry (map in Figure 3 left). The long fragments deriving from the same breakage points (767 and 758 nucleotides) are present, unresolved, near the 914-bp unbroken linear segment (empty arrow, lane 1). Lanes 3 and 4 show that nicked circular DNA (obtained by partial ligation and prepared by recovery from the gels as described) displays only one of the two fragile sites (position 147). In contrast, neither site is fragile in the covalently closed circular T_{-6} topoisomer.

The specific sites that appear to be sensitive to the single strand-specific endonuclease, P1, in the T_{-6} topoisomer are mapped in lane 6. At the *GAL1* end, each of these sites is relevant for the transcription process: the UAS (cut position, 405 ± 4), the TATA sequence (cut position, 675 and 678–682 ± 2), the region that encompasses the RNA initiation site (RIS) (cut position, 750, 754, 760, 767 ± 1) (Figure 3, lane 6).

The *in vivo* UAS function has been localized in base pairs 388–421, the *GAL1* TATA sequence is at 676–681, and the *in vivo* *GAL1* RISs are at 745, 755, 760, 764 and 765 (see Introduction).

The effect of increasing torsional stress on the formation of PHS at the *GAL10* end is shown in Figure 4: lanes 1–4 show that increased sensitivity gradually appears in passing from T_{+2} to T_{-6}. The three sites near the top, which are the most sensitive, map in proximity to (431 ± 4), or inside the UAS (422 and 405 ± 4) (see schematic drawing to the right of lane 4, Figure 4). The other major sensitive sites (251 ± 2, 201 ± 1) do not match exactly with the TATA sequence (present at positions 263–268) or with the RIS region (from 153 to 186) (map in Figure 3). The conformational alteration identified by P1 sensitivity on this sequence is already present at low stress (T_{+2}), lane 1) and increases as a function of increasing linking deficiency (lane 4).

At the *GAL1* end (lanes 5–8 of Figure 4), increased sensitivity also appears as a function of increased torsional stress. In this case the supercoiling dependence of conformational alterations is even more marked (i.e. for the UAS and the TATA sequence).

One should note that both segments of the *GAL1–GAL10* region are present on the same topological domain and are analyzed together (the analysis only differs in the final restriction site, the same topoisomers are treated with the same concentrations of P1 under the same conditions). The difference in behaviour of the P1 sensitivity of the common site (the UAS) is therefore necessarily only apparent; it is due to the fact that the UAS is placed, in the two analyses, at different distances from the labelled extremity and is differently competed by cuts in the sequence between the UAS and the labelled end. This makes the sensitivity of detection of P1 sensitivity lower at the UAS of the *GAL1* end.

That effects of this kind do indeed occur at the *GAL1* end is shown in Figure 4 (lanes 9–14, and lanes 15–19). The experiment shows the PHS as a function of increasing concentrations of P1. It reveals that, after a plateau range of concentrations (lanes

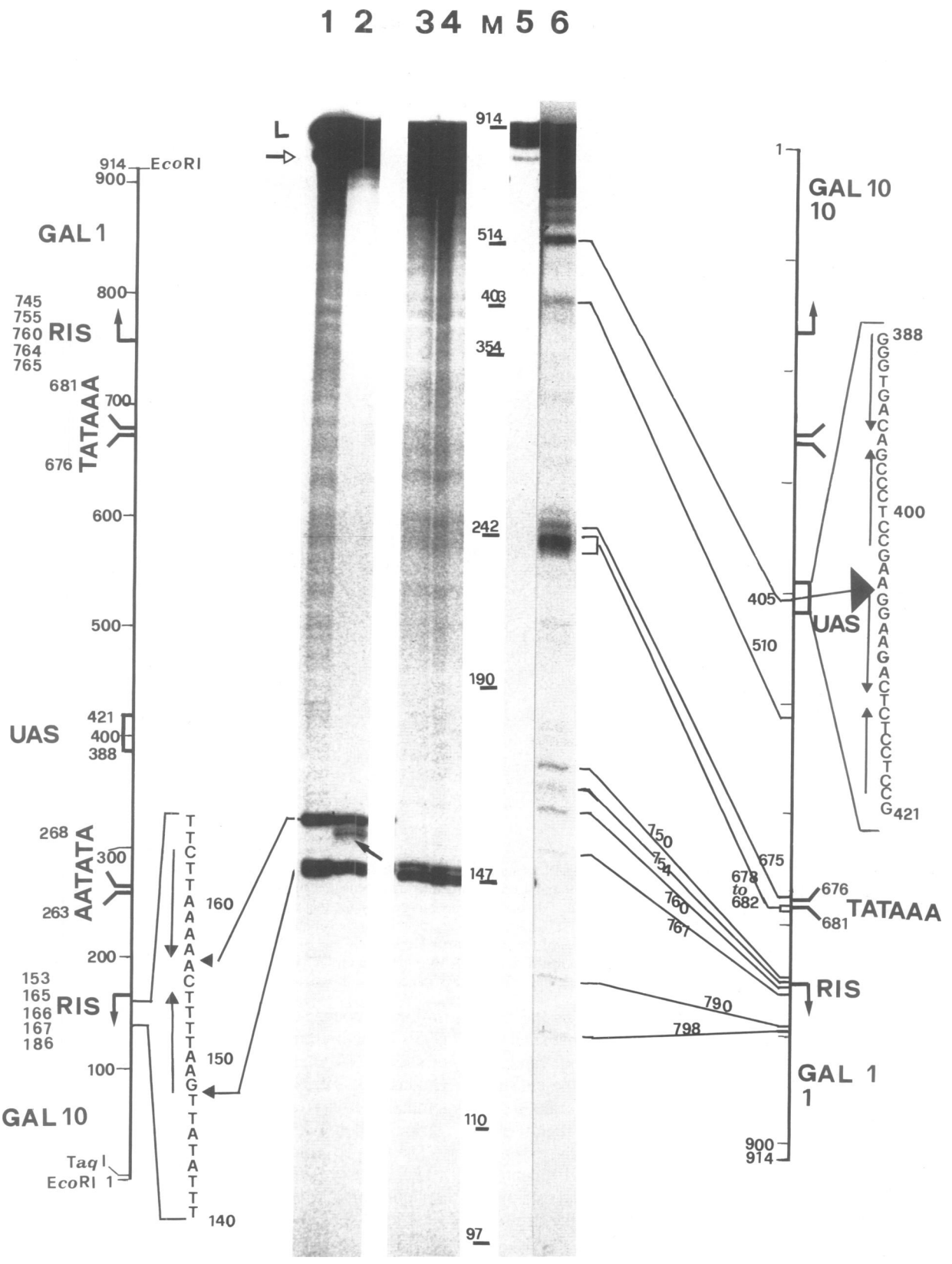

Fig. 3. Localization of PHS in linear, nicked and supercoiled *GAL1–GAL10* DNA. **Lanes 1** and **2:** *GAL1–GAL10* linear DNA terminally labelled at the *Eco*RI extremities was treated with 0.08 U of P1 (2 min at 37°C) and processed as described in Materials and methods **(lane 2). Lane 1** is the untreated control. **Lanes 3** and **4:** untreated and P1-treated (as above) nicked form, obtained by partial ligation and recovered from agarose gel. **Lanes 5** and **6:** topoisomer T_{-6} purified from a ligation at 10°C in the presence of 1.05 μg/ml of EtdBr. **Lane 5,** untreated; **lane 6,** P1-digested. Secondary restriction to orient the cuts: *Taq*I. M = marker lane consisting of a *Hpa*II digest of pUR250 DNA. The corrections necessary to pass from fragment sizes to map positions are detailed in Materials and methods. The two inverted repeats in the UAS are between 388 and 420 (Yocum *et al.*, 1984; West *et al.*, 1984). Numbers on the outer side of the map at the right refer to the positions of TATA and UAS identified *in vivo* (see Introduction), the numbers on the inner side refer to the map position of the P1 produced fragments.

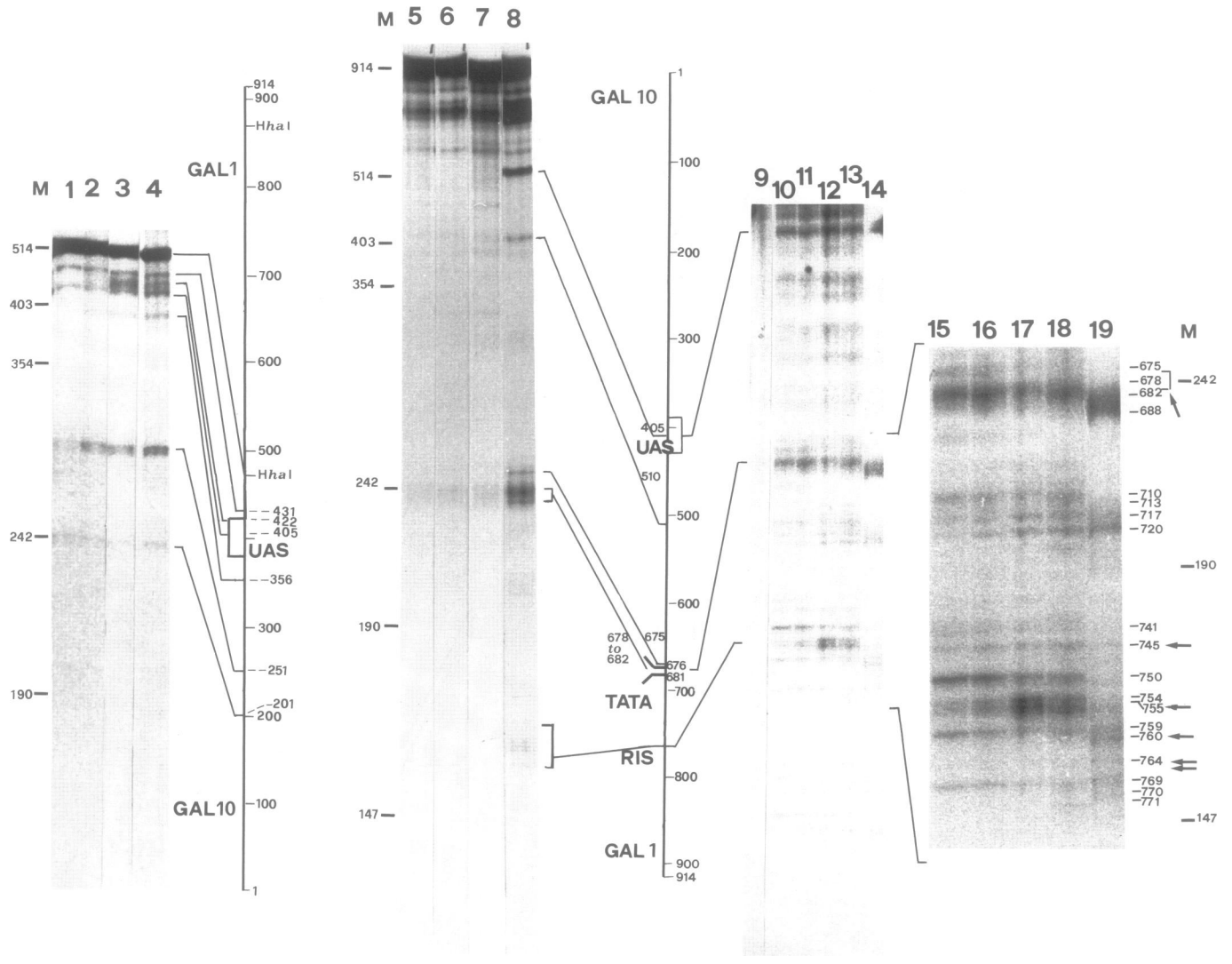

Fig. 4. PHS in function of superhelical density and of increasing P1 treatment. **Lanes 1–4:** *GAL10* end. P1 sites (from left to right) on T_{+2}, T_{-3}, T_{-4} and T_{-6} obtained by ligation at 10°C in the presence of 0, 0.75, 0.9 and 1.05 μg/ml EtdBr and recovered in pure form by gel electrophoresis. P1 digestion and electrophoretic analysis as described. Secondary restriction: *Hha*I. For the corrections necessary for mapping the PHS see Materials and methods. **Lanes 5–8:** *GAL1* end; topoisomers (from left to right): T_{+2}, T_{-3}, T_{-4} and T_{-6}. Secondary restriction: *Taq*I. Numbers on the left side of the map between **lanes 8** and **9** refer to P1-sensitive sites; numbers on the right are coordinates of the map. **Lanes 9–14** show the P1 cuts on T_{-6} produced by 0.008, 0.08, 0.16, 0.32, 0.8 and 2.4 U of P1. **Lanes 15–19:** enlargement of the data shown in **lanes 10–14**. Numbers on the right of **lane 19** show the positions of the P1 sites, the upper arrowed bracket shows the position of the TATA sequence, the lower arrows indicate the *in vivo* RIS.

15–18), severe enzymatic treatment (lane 14) causes the disappearance of the PHS localized further upstream from the labelled end, without provoking smeared digestion at random non-specific sites. Molecules cut twice only reveal the cut that is proximal to the label. This is also evident at local scale in the enlargement (compare lanes 18 and 19): in the RIS region, for instance, the lower positions become more evident at the expense of the higher ones. The arrows on the right of lane 19 identify the positions at which *in vivo* RNA synthesis starts and establish the correspondence between altered conformation in the stressed domain and the *in vivo* functional sites.

In vitro transcription of different topoisomers by purified yeast RNA polymerase II

We have previously developed (Carnevali *et al.*, 1982; Di Mauro *et al.*, 1985) a transcription assay for the detection of *in vitro* initiation events with purified eukaryotic (yeast) RNA polymerase II. Ternary transcription complexes (TTC) are formed between purified yeast RNA polymerase II and DNA in the presence of limiting amounts of enzyme and Mg^{2+} (binding). A pulse of NTPs in the presence of Mn^{2+} (elongation) allows the formation of short RNA chains. Agarose gel electrophoresis (Chelm and Geiduschek, 1979) of TTC allows quantitative evaluation of the template activity (and mapping of the initiation regions) under various topological conditions (Carnevali *et al.*, 1983).

The 914-bp *GAL1–GAL10* intergenic region was ligated at 10°C, in the presence of increasing EtdBr concentrations, yielding a population of topoisomers identical to the one shown in Figure 1c. Figure 5a shows the results of *in vitro* transcription (TTC) performed on these DNAs. A sharp activation of transcription takes place between the populations of topoisomers T_{-5} and T_{-6}. Figure 5b shows a more detailed analysis of the region of transition and reveals that activation of templates is associated with the appearance of T_{-6} and T_{-7}. When temperature was used as the varying parameter (in the presence of a fixed amount of EtdBr) to produce topoisomers, the same correlation between template activity and presence of T_{-6}, T_{-7} was found (not shown).

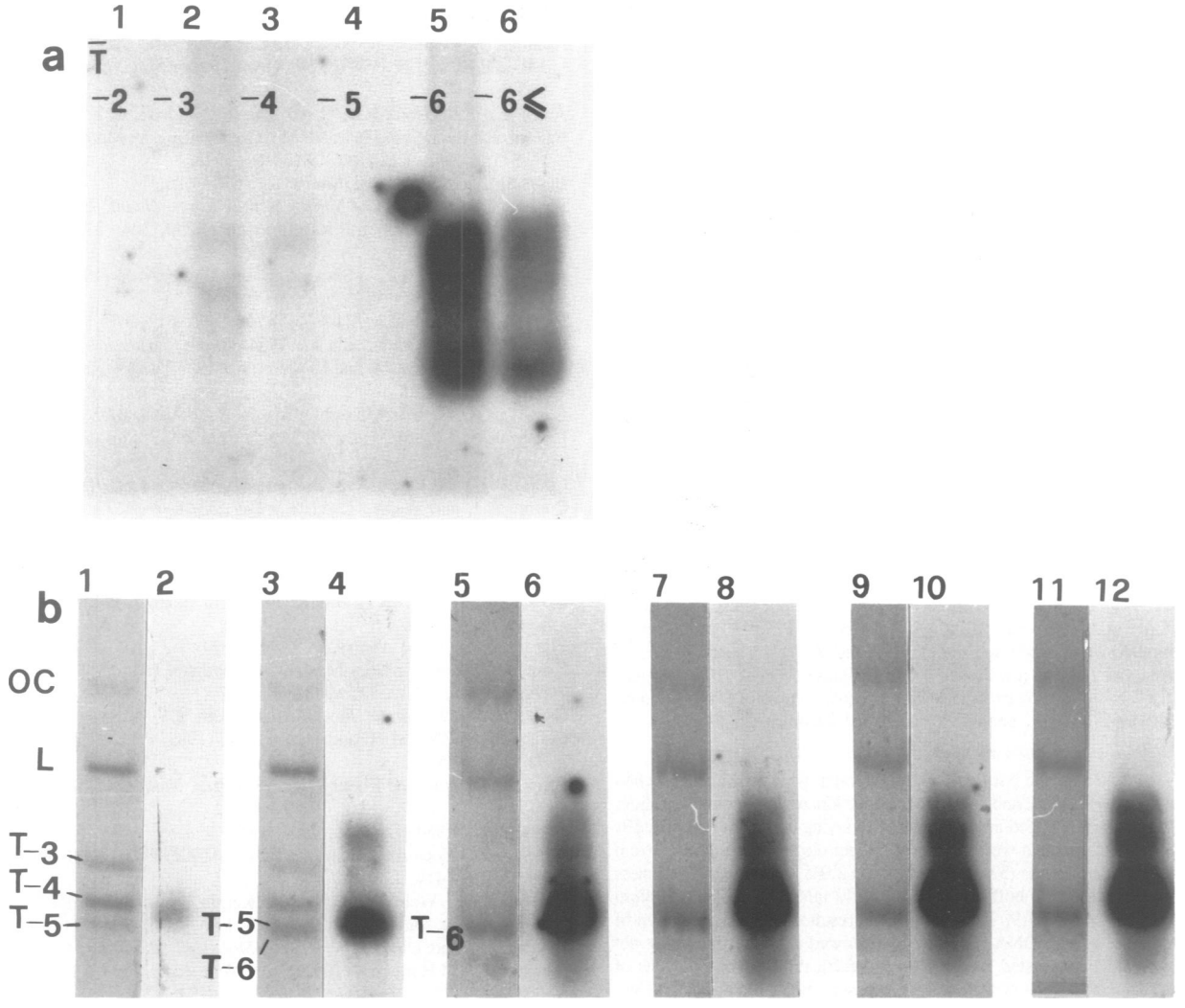

Fig. 5. Analysis of ternary transcription complexes formed on different topoisomeric populations. TTC were formed and analyzed as described (Carnevali *et al.*, 1982, 1984). **Panel a** shows the autoradiogram of the transcription obtained reacting 1 μg of 914-bp DNA circularized at 10°C. Ligation was in the presence of 0.5, 0.6, 0.75, 0.9, 1.05 and 2 μg/ml EtdBr (from **lane 1** to **lane 6**) producing topoisomers with τ = 2, 3, 4, 5, 6, ≥6 as indicated on top of each lane. **Panel b** shows a detail of the transition region: topoisomers were formed at 10°C in the presence of 0.9, 1.0, 1.05, 1.15, 1.25 and 1.4 μg/ml EtdBr, producing the populations reported alongside the autoradiograms. **Lanes 1, 3, 5, 7, 9** and **11** fluorograms. **Lanes 2, 4, 6, 8, 10** and **12** autoradiograms.

Discussion

We have shown that the sites undergoing conformational alteration in the circularized *GAL1–GAL10* intergenic region under torsional stress correspond to the three positions identified as relevant for the onset of *in vivo* transcription of *GAL1* (UAS, TATA-box and RIS) (Guarente, 1984; Brent and Ptashne, 1984; West *et al.*, 1984; Yocum *et al.*, 1984).

The topology of a DNA domain appears therefore to be a basic parameter for the attainment of a defined conformation of nucleotide sequences. In the case described here the sequences are those involved in the *in vivo* regulation of transcription. The major problem facing the evaluation of the physiological meaning of the topological variations found is that no data are available or are in reach on the exact topology *in vivo* of the sequences analysed. The observation that the stressed domain reaches template activity only at the highest level of supercoiling (in correspondence with the attainment of marked alternative conformations on the UAS, the TATA sequence and the RIS in the *GAL1* moiety) should only be considered a correlation and not a proof of a cause–effect relationship between topology and transcription.

It has been postulated that the activity of regulatory regions is based on 'conformational information' (Rich, 1983). According to this model, the difference between active and inactive conformation relies on the topological behaviour of the components of the system. The experimental system described here defines that a threshold of superhelical density ($-\sigma$ = 0.068) exists beyond which DNA changes dramatically both the conformational information of the UAS, the TATA and the RIS sequences and the *in vitro* template activity.

Materials and methods

Materials

P1 endonuclease was obtained from Pharmacia. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs and Boehringer. EtdBr was purchased from Sigma, radiochemicals from NEN. Topoisomerase I was purified from frozen chicken erythrocytes according to Bina-Stein *et al.* (1976). Yeast RNA polymerase II purified to homogeneity (Lescure *et al.*, 1981) was a kind gift of J.Huet and A.Sentenac (Saclay, France).

The recombinant plasmid pSc4816 (Yocum *et al.*, 1984) contains a 914-bp

*Eco*RI–*Ava*I fragment which covers the region between *GAL1* and *GAL10* cloned with an *Eco*RI linker into the *Eco*RI site of pBR325. This plasmid was a gift of J.Hopper and S.Johnston. The sequence of the 914-bp insert is in Yocum *et al.* (1984). The same identical sequence has also been published by Johnson and Davis (1984). The numbering reported by Yocum *et al.* (1984) has been followed throughout the present paper. DNA was purified according to a standard procedure (Ballario *et al.*, 1981).

P1 treatment

This was carried out as detailed for similar experiments with S1 (Carnevali *et al.*, 1984). The conditions of the reaction (~100 ng of supercoiled DNA, 0.08 U of P1, 37°C, 1 min unless otherwise specified, in 50 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.9) were planned to introduce single-stranded nicks and yielded (as judged by agarose gel electrophoresis) 80% nicked, 10% linear and 10% unmodified supercoiled DNA.

Topoisomerase I assay

0.5 U of enzyme (defined in Keller, 1975a, 1975b) were reacted in 50 μl of 0.1 M NaCl, 10 mM Tris-HCl, pH 7.8 and 1 mM EDTA at 35°C for the time specified with ~100 ng of supercoiled DNA.

Circularization of DNA fragments

The procedure detailed by Schon *et al.* (1983) was followed with minor modifications: the 914-bp fragment was excised from the vector by *Eco*RI digestion, separated through agarose electrophoresis, recovered by electroelution, phenol extracted and alcohol precipitated. Circularization was carried out at low DNA concentration (1 μg/ml, to avoid formation of concatamers) in the presence of the specified concentrations of EtdBr, at the specified temperature. The ligase reaction mixture (700 μl) contained 25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.2 mM spermidine, 0.5 mM ATP, 25 μg/ml of bovine serum albumin and 2 Weiss units/ml of T4 DNA ligase (Weiss, 1971) and was carried out for 2 h; stopped by extraction with an equal volume of Sevag solution, concentrated with 2-butanol, phenol extracted and alcohol precipitated.

Mapping the P1-hypersensitive sites

The *Eco*RI extremities of the purified 914-bp fragment were terminally labelled at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase before circularization. The fragments were circularized in the presence of varying concentrations of EtdBr at 2°C. The ligated products were separated by gel electrophoresis at 2 V/cm for 18 h in a vertical apparatus (37 × 18 × 0.3) on 2.0% agarose, at 20°C unless otherwise specifiifed, with a buffer composed of 40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.9. These conditions resulted in good resolution of topoisomers of the 914-bp DNA fragment and allowed isolation by cutting out bands of single uncontaminated topoisomers. Identification of the positions of bands was achieved by brief autoradiographic exposure of the wet gel. After electroelution and purification, the isolated topoisomers were treated with P1, phenol extracted, alcohol precipitated and treated with a secondary restriction endonuclease to allow directional mapping of the P1 cutting sites. For the analysis from the *GAL1* extremity the *Taq*I site (cut at position 5 in the upper strand, at position 7 in the lower) was used. The resulting linear DNA fragments were denatured (90% formamide, 90°C for 30 s) and analyzed on thin 6% polyacrylamide gels containing 8 M urea. Gels were exposed to Cronex film (Du Pont) using intensifier screens. The lengths of the single-stranded DNA fragments produced by P1 on one side and by restriction on the other, were identified by comparison with reference ladders composed of a complete *Hpa*II digest of pUR250. In passing from the size identified in the gel to the position in the map, a correction of −6 is necessary (the map being numbered from the *Eco*RI site); the resulting value was subtracted from 914 (the conventional numbering is from the *GAL* to extremity). The ambiguity resulting from this procedure is of plus or minus 1 nucleotide depending on whether the P1 cut is on the upper or on the lower strand. The position of the *Hha*I site used for mapping from the *GAL10* extremity is 870; an additional *Hha*I site is at 470 (see Figure 3). For a localization of the PHS on this extremity, the sizes obtained were subtracted from 44 (distance between the *Hha*I site at 870 and the *Eco*RI site).

Ternary transcription complexes

These were obtained and analyzed as described by Carnevali *et al.* (1982, 1984). Experimental details are given in the legend to Figure 5.

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