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# Preferential, cooperative binding of DNA topoisomerase II to scaffold-associated regions

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**DNA elements termed scaffold-associated regions (SARs) are AT-rich stretches of several hundred base pairs which are known to bind specifically to nuclear or metaphase scaffolds and are proposed to specify the base of chromatin loops. SARs contain sequences homologous to the DNA topoisomerase II cleavage consensus and this enzyme is known to be the major structural component of the mitotic chromosome scaffold. We find that purified topoisomerase II preferentially binds and aggregates SAR-containing DNA. This interaction is highly cooperative and, with increasing concentrations of topoisomerase II, the protein titrates quantitatively first SAR-containing DNA and then non-SAR DNA. About one topoisomerase II dimer is bound per 200 bp of DNA. SARs exhibit a *Circe* effect; they promote in *cis* topoisomerase II-mediated double-strand cleavage in SAR-containing DNA fragments. The AT-rich SARs contain several oligo(dA)·oligo(dT) tracts which determine their protein-binding specificity. Distamycin, which is known to interact highly selectively with runs of A·T base pairs, abolishes the specific interaction of SARs with topoisomerase II, and the homopolymer oligo(dA)·oligo(dT) is, above a critical length of 240 bp, a highly specific artificial SAR. These results support the notion of an involvement of SARs and topoisomerase II in chromosome structure.**

*Key words:* chromosome/cooperativity/SAR/scaffold/topoisomerase II

## Introduction

DNA elements termed scaffold-associated regions (SARs) are AT-rich sequences several hundred base pairs long which are known to bind specifically to nuclear or metaphase scaffolds and are proposed to form the base of chromatin loops (for a review see Gasser and Laemmli, 1987). SARs (sometimes called MARs) were originally identified in *Drosophila* cell lines (Mirkovitch *et al.*, 1984) and have since been characterized in several organisms including human and yeast (Cockerill and Garrard, 1986a; Käs and Chasin, 1987; Amati and Gasser, 1988; Phi-Van and Strätling, 1988).

An extensive characterization of SARs has been made in *Drosophila melanogaster*, with nearly 20 SARs mapped in RNA polymerase II-transcribed domains extending over 400 kb of the genome (Mirkovitch *et al.*, 1987). Several of the *Drosophila* SARs are localized near enhancers or other regulatory elements or at the boundaries of functional domains (Gasser and Laemmli, 1986), and similar observations have been made in other organisms (Cockerill and Garrard, 1986a; Phi-Van and Strätling, 1988). SARs appear evolutionarily conserved as they bind specifically to nuclear scaffolds across species boundaries (Cockerill and Garrard, 1986b; Mirkovitch *et al.*, 1988).

Whilst attempting to isolate proteins which bind specifically to SARs we observed that histone H1 interacts highly selectively with SAR-containing DNA (Izaurralde *et al.*, 1989). Our experiments have identified SARs as *cis*-acting sequences that nucleate cooperative H1 assembly onto flanking non-SAR DNA. The AT-rich SARs contain many dA·dT runs, including the arrays previously described as A and T boxes (Gasser and Laemmli, 1986). Experiments with simple DNA polymers have shown that these oligo(dA)·oligo(dT) tracts determine the specificity of SARs. The homopolymer oligo(dA)·oligo(dT) is, above a critical length of 130 bp, a highly specific nucleator of H1 assembly (Izaurralde *et al.*, 1989). The oligo(dA)·oligo(dT) tracts are also involved in the specific interaction of SARs with nuclear scaffolds as suggested by experiments with distamycin. This cytotoxic drug, which is known to interact highly selectively with dA·dT base pairs (Van Dyke *et al.*, 1982; Fox and Waring, 1984), abolishes the specific interaction of SARs both with scaffolds and histone H1 (Käs *et al.*, 1989). It is not clear whether SARs can nucleate H1 binding *in vivo*, but it is attractive to consider the possibility that SARs might determine, via a regulated, preferential H1 assembly, the long-range conformation of chromatin domains and consequently set up the potential transcriptional repertoire of the cell (Izaurralde *et al.*, 1989).

In addition to the oligo(dA)·oligo(dT) tracts, SARs contain sequences related to the topoisomerase II cleavage consensus (Cockerill and Garrard, 1986a; Gasser and Laemmli, 1986). DNA topoisomerase II mediates interconversions between different topological states of DNA through transient double-strand breaks and rejoining. Topoisomerase II can relax both positive and negative supercoils, catenate and decatenate DNA rings, and unknot knotted DNA (reviewed by Wang, 1985). The enzyme is also required for chromatin assembly and packaging as demonstrated by the sensitivity of *Xenopus in vitro* nuclear reconstitution systems to topoisomerase II inhibitors (Newport, 1987). In yeast, available evidence suggests that topoisomerase II is required for the final stages of chromosome condensation (Uemura *et al.*, 1987) and to untangle sister chromatids during anaphase (Holm *et al.*, 1985; Uemura and Yanagida, 1986).

Topoisomerase II is the major non-histone component of the metaphase scaffold and immunolocalization of this enzyme in gently expanded metaphase chromosomes has revealed that it is found along the longitudinal central axis spanning the chromatid (Earnshaw and Heck, 1985; Gasser *et al.*, 1986). In more compact chromosomes the scaffolding is helically folded with sister chromatids having predomi-

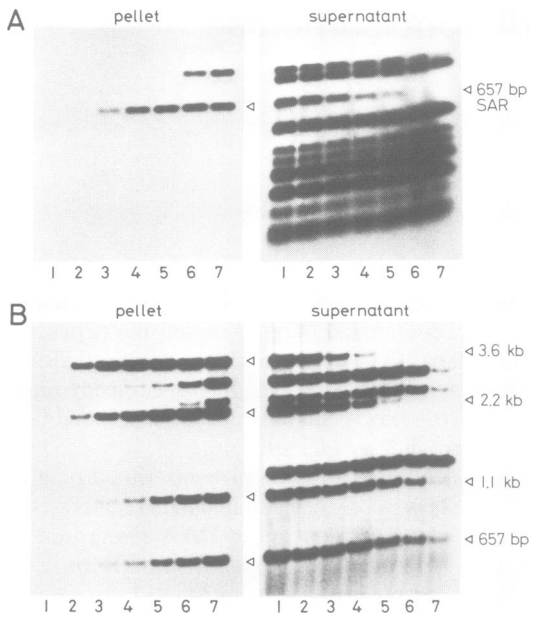


**Fig. 1.** Preferential aggregation of a SAR-containing fragment by topoisomerase II. **Panel A**: plasmid pUL402, which contains the 1.3 kb *Hin*dIII−*Eco*RI spacer fragment from the *Drosophila* histone gene cluster was digested with *Eco*RI and *Hin*fI, end labeled and used as a probe. The histone SAR is located on the 657 bp fragment indicated by the arrow (Mirkovitch *et al.*, 1984). This probe was incubated with 500 ng of sonicated salmon sperm DNA and increasing amounts of topoisomerase II in TEN buffer at 30°C for 30 min. Samples were spun in a microfuge for 10 min to collect the insoluble aggregates. DNA samples from the pellet and supernatant fractions were applied to a 1.4% agarose gel and examined by autoradiography. For **lanes 1−7**, 0, 125, 250, 375, 500, 750 and 1000 ng of topoisomerase II were used respectively. **Panel B**: the effect of DNA length flanking the histone SAR on the affinity for topoisomerase II was studied using end-labeled probes containing the 657 bp histone SAR flanked at one end by increasing lengths of pSP64 vector DNA. The probe mixture was prepared by mixing the following digests of clone pSP64-34: *Eco*RI and *Hin*fI, *Eco*RI and *Sph*I, *Eco*RI and *Bgl*I and *Eco*RI alone. This probe mixture was incubated with 500 ng of sonicated salmon sperm DNA and 0 (**lane 1**), 150 (**lane 2**), 200 (**lane 3**), 250 (**lane 4**), 375 (**lane 5**), 500 (**lane 6**) and 750 ng (**lane 7**) of topoisomerase II. The DNA in the pellet and supernatant fractions was analyzed by agarose gel electrophoresis. SAR-containing fragments and their sizes are indicated by the arrows.

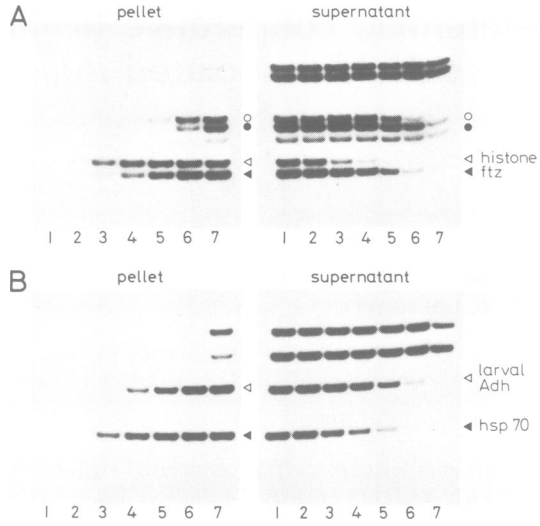


**Fig. 2.** Aggregation assays with different SAR DNA fragments. The procedure and concentrations of topoisomerase II used are similar to those described in the legend to Figure 1A. Arrows indicate the position of the SAR-containing fragments. The DNAs from pellet and supernatant fractions obtained at different topoisomerase II concentrations were analyzed by agarose gel electrophoresis. **Panel A**: clone DM506, which contains the histone large repeat unit (5 kb *Hin*dIII fragment) of *Drosophila* inserted into the *Hin*dIII site of pBR322, was digested with *Eco*RI, *Xho*I and *Hin*dIII. The SAR (indicated by the open arrow) is located on a 1.3 kb *Hin*dIII−*Eco*RI fragment (Mirkovitch *et al.*, 1984). Fushi tarazu clone pFKH1 (Hiromi *et al.*, 1985) was digested with *Eco*RI and *Hin*dIII. The *ftz* SAR is located on a 1.2 kb *Eco*RI fragment indicated by the filled arrow. The histone and *ftz* probes were mixed in the same reaction mixtures and pellet and supernatant fractions were examined by agarose gel electrophoresis. Two histone and *ftz* non-SAR fragments (indicated by the open and filled circles respectively) were found to aggregate slightly better than vector non-SAR fragments. **Panel B**: heat-shock gene clone 122-1 from locus 87A7 (Goldschmidt-Clermont, 1980) was digested with *Eco*RI and *Xho*I. The SAR is located on a 1.27 kb intergenic fragment indicated by the filled arrow (Mirkovitch *et al.*, 1984). Clone pGAC1.9 which contains the larval SAR of the alcohol dehydrogenase gene, was digested with *Eco*RI. The SAR (open arrow) is located on a 1.9 kb *Eco*RI fragment (Gasser and Laemmli, 1986).

nantly opposite helical handedness (Boy de la Tour and Laemmli, 1988). These findings as a whole and the observation that topoisomerase II preferentially binds and cleaves the SAR of the divergent *Drosophila hsp70* genes (Udvardy *et al.*, 1985, 1986; Sander *et al.*, 1987) suggest that it might be involved in SAR-mediated attachment of chromatin loops to the nuclear scaffold.

To address the question of how the soluble enzyme topoisomerase II might play such a structural role in chromosomes, we have undertaken a detailed study of the interaction of this protein with SARs *in vitro*. Results of our experiments show that topoisomerase II preferentially and cooperatively binds SAR-containing DNA fragments, which can be specifically recovered in insoluble protein−DNA aggregates. Drug-induced cleavage experiments confirm the cooperative nature of this selective interaction, and establish that *cis*-acting SARs promote greatly increased topoisomerase II cleavage in flanking non-SAR sequences. This *Circe* effect of SARs is abolished in the presence of the drug distamycin, suggesting, as is the case for histone H1−SAR interactions, that dA·dT tracts are major determinants of selective topoisomerase II binding to DNA.

## Results

### *Topoisomerase II specifically aggregates SARs*

All SARs characterized in the *Drosophila* genome contain sequences related to the topoisomerase II cleavage consensus (Gasser and Laemmli, 1986). The SAR located in the intergenic region of the *Drosophila hsp70* genes at locus 87A7 (Mirkovitch *et al.*, 1984) is known to contain both preferential binding and cleavage sites for topoisomerase II (Sander *et al.*, 1987). Since a number of observations suggest that topoisomerase II might play a role in chromosome structure, we have addressed the question of how this soluble protein might be involved in the long-range organization of chromatin by studying its interaction with SARs *in vitro*.

In these studies, we have used highly purified topoisomerase II, isolated from a *Schizosaccharomyces pombe* strain harboring the topoisomerase II gene on a multicopy plasmid (Uemura and Yanagida, 1986). In our standard binding assay, end-labeled restriction fragments are incubated with purified topoisomerase II in the presence of sonicated salmon

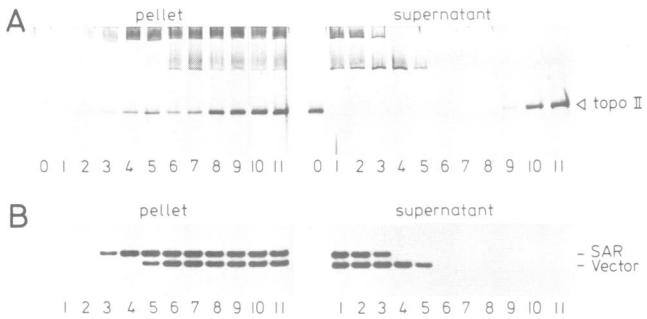


**Fig. 3.** Cooperative binding of topoisomerase II to DNA fragments. Vector pSP64 and clone pSP64-34 which contains the 657 bp histone SAR were linearized by cutting at a unique *Eco*RI site. 200 ng of each fragment (containing trace amounts of end-labeled DNA) were mixed and incubated in 100 μl of buffer TEN with 0, 100, 200, 400, 600, 800, 1000, 1200, 2000, 3000 and 4000 ng of topoisomerase II (**lanes 1–11** respectively). Salmon sperm DNA was omitted to avoid background staining in protein gels. After a 30-min incubation at 30°C, the samples were divided into two aliquots and centrifuged. One set was used for protein gel electrophoresis (**panel A**) and the other for DNA agarose gel electrophoresis (**panel B**). **Panel A**: the silver-stained SDS-polyacrylamide gel of the pellet and supernatant fractions is shown. **Lane 0** contained 500 ng of topoisomerase II in a 50 μl reaction mixture without added DNA. Note that in the other lanes the amounts of topoisomerase II partitioning into the pellet and supernatant fractions represent half of the protein inputs indicated above. Some of the topoisomerase II in the pellet fractions (particularly in **lanes 6** and **7**) was lost during pellet washes. The two smeared bands visible in the other lanes above the topoisomerase II band are the pSP64-34 (upper) and pSP64 (lower) DNA fragments. **Panel B**: the other 50 μl aliquots were treated with 0.5% SDS and 0.5 mg/ml proteinase K (final) at 60°C for 60 min and electrophoresed on a 0.8% agarose gel. The autoradiograph of the gel is shown.

sperm competitor DNA. In intial experiments, we noted the formation of protein–DNA aggregates which could be collected by centrifugation, and we used this simpler assay rather than a filter-binding technique in subsequent experiments (see Discussion). Following incubation for 30 min at 30°C, samples were spun down in a microfuge and the DNA fragments in the pellet (P) or supernatant (S) fractions were analyzed by gel electrophoresis.

Plasmid clone pUL402, which contains the SAR from the *Drosophila* histone gene cluster (Mirkovitch *et al.*, 1984), was digested with *Eco*RI and *Hin*fI, end-labeled, and incubated with increasing amounts of topoisomerase II. The results shown in Figure 1A, demonstrate the selective aggregation of the 657 bp SAR (indicated by the arrow) into the pellet fraction at a topoisomerase II:DNA input ratio of 0.5–1.0 (w/w). Near the optimal protein:DNA ratio of 1.0 (lane 5) it is possible to fractionate quantitatively the SAR and non-SAR fragments into the pellet and supernatant respectively.

We tested other *Drosophila* SARs for a similar preferential interaction with topoisomerase II. Figure 2 shows the results of binding experiments for the SAR associated with the larval promoter of the alcohol dehydrogenase (*Adh*) gene, the 5′ SAR from the *fushi tarazu* (*ftz*) gene, the SAR associated with the heat shock (*hsp70*) genes from locus 87A7, and the SAR from the histone gene cluster (Mirkovitch *et al.*, 1984; Gasser and Laemmi, 1986). Restriction fragments containing these SARs are 1.9 kb in length for the larval *Adh* SAR, 1.2 kb for the *ftz* SAR, 1.27 kb for the *hsp70* SAR, and 1.3 kb for the histone SAR. All these SARs (indicated by the arrows) were efficiently aggregated by topoisomerase II while non-SAR fragments remained in the supernatant fraction up to topoisomerase II:DNA input ratios of 1.0 (Figure 2A and B, lane 6). We observed SAR-specific binding at protein:DNA input ratios ranging from 0.2 to 0.75 for the histone SAR (Figure 2A, lanes 2–6), from 0.3 to 0.75 for the *ftz* and *hsp70* SARs (Figure 2A and C, lanes 3–6), and from 0.4 to 0.75 for the larval *Adh* SAR (Figure 2B, lanes 4–6). We also tested the SARs associated with the adult promoter of the *Adh* gene and with the glue-protein *Sgs4* gene (Gasser and Laemmli, 1986) and observed weaker binding (data not shown).

### *The size of the flanking non-SAR DNA affects the binding affinity*

The optimal DNA:protein ratio which results in specific and quantitative precipitation varies for different SARs and, for a given SAR, depends on the length of the flanking DNA. To illustrate this observation, we prepared different probes consisting of the 657 bp histone SAR alone or flanked at one end by increasing extents of vector DNA, yielding SAR-containing fragments of 1.1, 2.2 and 3.6 kb respectively. This end-labeled probe mixture was tested for preferential association with different amounts of topoisomerase II in the presence of a fixed concentration of salmon sperm competitor DNA. The gel in Figure 1B, shows that, at low protein:DNA input ratios, topoisomerase II aggregates the larger SAR-containing fragments first; the smaller fragments are then progressively precipitated as more topoisomerase II is added. Thus, the 3.6 kb SAR-containing fragment is quantitatively aggregated at a protein:DNA ratio of 0.75, while about twice as much topoisomerase II is required to precipitate the 657 bp SAR alone (Figure 1B, lanes 5 and 7). Not surprisingly, at the highest topoisomerase II concentration used, some non-specific aggregation is noted for the larger (>2 kb) non-SAR fragments but not for the smaller control fragment (Figure 1B, lanes 6 and 7).

The preferential interaction of topoisomerase II with SARs must be due to energetically favorable interactions with this DNA. The free energy gain of the complex appears to be cumulative and propagated into the flanking DNA since increasing the length of the non-SAR DNA lowers the amount of enzyme required for selective and quantitative aggregation of the SAR-containing DNA. This interpretation, if correct, suggests that topoisomerase II binds DNA cooperatively.

### *Topoisomerase II binds SAR-containing DNA cooperatively*

We demonstrated the higher cooperative nature of topoisomerase II binding to SAR-containing DNA as follows. We prepared a probe mixture consisting of equal weights of plasmid pSP64-34, which contains the 657 bp histone SAR, and of the pSP64 vector alone; these plasmids were end-labeled following linearization with *Eco*RI. This probe mixture was incubated with increasing amounts of topoisomerase II. After a 30 min incubation at 30°C, the samples were divided into two aliquots. Following centrifugation, one set was analyzed by agarose gel electrophoresis, the other by SDS–PAGE, to follow the fate of both DNA and protein.

Under these conditions of competition, the 3.6 kb SAR-containing fragment was progressively aggregated as more topoisomerase II was added, until quantitatively precipitated (Figure 3B, lanes 2–4). Only then did precipitation of the 3.0 kb pSP64 vector fragment occur (lanes 5 and 6).

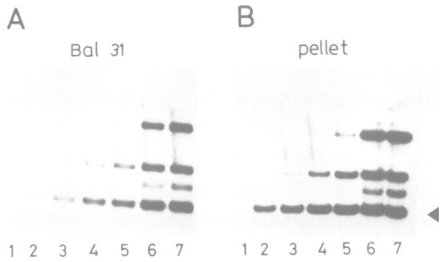


**Fig. 4.** Topoisomerase II bound DNA fragments are protected against degradation by *Bal*31. *Drosophila* histone clone DM506 was digested and end labeled as described in the legend to Figure 2A. The probe was mixed with 500 ng of salmon sperm competitor DNA in 100 μl of buffer B and incubated with 0, 300, 400, 500, 600, 1200 and 1800 ng of topoisomerase II (**lanes 1–7** respectively) for 30 min at 30°C. The samples were then divided into two 50 μl aliquots. One set was digested with *Bal*31 and the other was spun down and fractionated into pellet and supernatants. *Bal*31-digested samples (**panel A**) and the corresponding pellet fractions (**panel B**) were analyzed on an agarose gel. The 1.3 kb histone SAR fragment is indicated by the arrow.

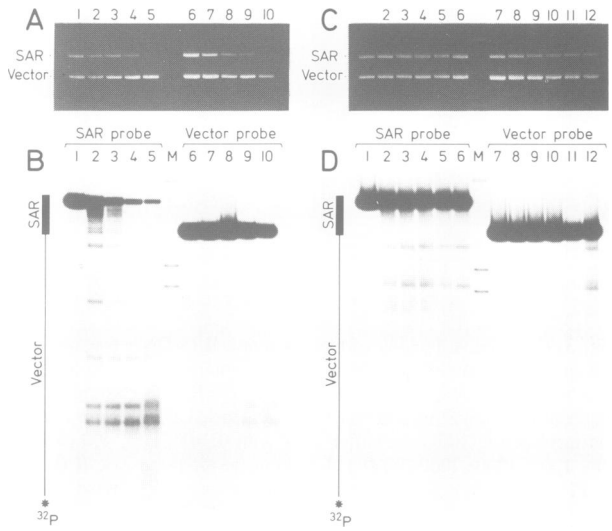


**Fig. 5.** Preferential cleavage of SAR-containing DNA: the *Circe* effect. DNA mixtures consisting of equal weights of vector pSP64 (Vector probe) and pSP64-34 (SAR probe) linearized with *Eco*RI were prepared and incubated with increasing amounts of topoisomerase II. After an incubation time of 5 min, the drug VM26 was added and incubation continued for an additional 5 min (see also Materials and methods). Reactions were terminated by addition of SDS. The DNA samples were analyzed on agarose gels after purification. In each mixture, only one of the probes (SAR or Vector) carried a radioactive label at their common end (see Materials and methods) as indicated above the gel panels. Lanes marked 'M' contained mol. wt standards. **Panel A**: photograph of the gel stained with ethidium bromide, showing gradual cleavage of the SAR probe. Samples contained 0, 50, 100, 200 and 300 ng of topoisomerase II (**lanes 1–5** and **6–10** respectively). **Panel B**: autoradiograph of the gel shown in panel A. The SAR probe is labeled in **lanes 1–5** and the Vector probe is labeled in **lanes 6–10**. A similar experiment was performed without added VM26. The stained gel is shown in **panel C**, the autoradiograph in **panel D**. Samples contained 0, 100, 200, 300, 500 and 750 ng of topoisomerase II (**lanes 1–6** for the labeled SAR probe and **lanes 7–12** for the labeled Vector probe respectively).

The protein gel analysis for this experiment is shown in Figure 3A. Topoisomerase II alone did not form aggregates and remained in the supernatant fraction (Figure 3A, lane 0). In the presence of DNA and with increasing amounts of input topoisomerase II, all the added enzyme, as determined by silver staining, was recovered in the pellet fraction (lanes 2–8); although in this experiment some of the topoisomerase II in the pellet fractions was lost during washes, its absence in the supernatant clearly indicates quantitative binding to the DNA. Topoisomerase II was only recovered in the supernatant once added in excess (lanes 9–11), well after all the input DNA had been quantitatively precipitated. Since the totality of the protein added is titrated by the DNA at the lower protein:DNA input ratios, and since the fraction of the DNA recovered in the pellet is known, it is possible to calculate protein:DNA ratios in the aggregates. We estimate that, at saturation, approximately one topoisomerase II dimer is bound per 200 bp of DNA.

We also analyzed the distribution of topoisomerase II along DNA fragments using exonuclease *Bal*31. In the following experiment an end-labeled histone probe (see Figure 2A) was incubated with increasing amounts of topoisomerase II in the presence of a fixed concentration of competitor DNA. Following the usual incubation period, half of each sample was digested with *Bal*31 (Figure 4A) and the other half was fractionated by centrifugation (Figure 4B). In the absence of topoisomerase II all of the input probe was digested by *Bal*31 (Figure 4A, lane 1). With added topoisomerase II and at low protein:DNA input ratios, we observed a highly selective protection of a significant fraction of the SAR but not the non-SAR DNA (lanes 2–5). At higher topoisomerase II:DNA input ratios other fragments became protected from digestion by *Bal*31. Note that these samples were not fractionated by centrifugation prior to digestion with *Bal*31 and that the appearance of the SAR in the pellet corresponds to that of SAR fragments protected from *Bal*31 digestion (Figure 4, cf. panels A and B). Since the probe was end-labeled, this experiment with exonuclease *Bal*31 demonstrates selective topoisomerase II-dependent protection of the DNA ends, providing additional evidence for cooperative topoisomerase II binding along the entire length of DNA molecules.

The results of these experiments show that the histone SAR nucleates preferential assembly of topoisomerase II onto flanking non-SAR DNA. Only after the SAR-containing DNA has been fully loaded does the non-SAR fragment begin to bind the protein. Following complete titration of the latter fragment (about one dimer per 200 bp), free topoisomerase II is recovered in the supernatant.

### *The Circe effect of SARs*

To study this SAR-dependent nucleation phenomenon by a different method, we took advantage of the enzymatic activity of topoisomerase II and analyzed topoisomerase II cleavage reactions on non-SAR and SAR-containing DNA in the presence of the drug epipodophyllotoxin, VM26. This cytotoxic drug stabilizes the transient covalent protein–DNA intermediate in the topoisomerase II DNA cleavage reaction, thus introducing protein-linked double-stranded breaks in the DNA upon addition of protein denaturants (Chen *et al.*, 1984). It has been previously demonstrated that SARs contain preferential topoisomerase II cleavage sites both *in vivo* and *in vitro* (Udvardy *et al.*, 1985, 1986; Sander *et al.*, 1897). The SAR-dependent nucleation of topoisomerase II assembly onto non-SAR DNA suggests that SARs might serve to lure and retain the protein in flanking sequences, consistent with our previous proposal of a 'Circean scaffold' (Mirkovitch *et al.*, 1984). We asked whether SARs might exhibit such a *Circe* effect, i.e. increase topoisomerase II cleavage activity in flanking non-SAR DNA.

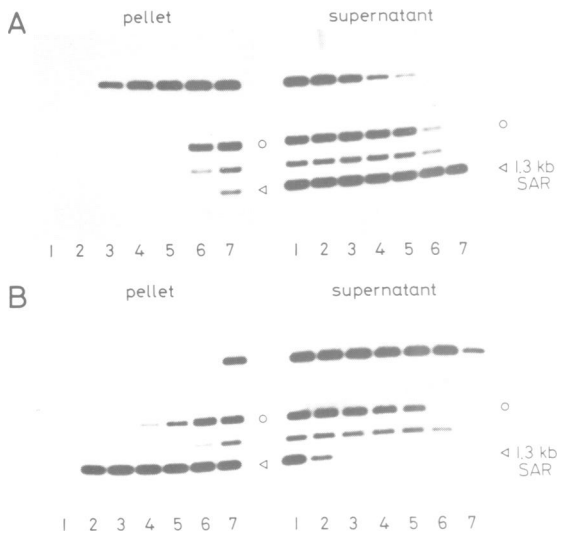


**Fig. 6.** Distamycin inhibits SAR-specific binding of topoisomerase II. **Panel A**: plasmid DM506, which contains the histone gene repeat cloned in pBR322, was digested with *Eco*RI, *Hin*dIII and *Xho*I and end labeled. The probe was mixed with 500 ng of sonicated salmon sperm DNA and treated with 20 $\mu$M distamycin. Topoisomerase II was then added and incubation was continued for 30 min. Samples contained 0, 100, 150, 200, 250, 500 and 750 ng of topoisomerase II (**lanes 1–7** respectively). DNAs from the pellet and supernatant fractions were analyzed by agarose gel electrophoresis. The 1.3 kb histone SAR is indicated by the arrow. The open circle indicates a 2.2 kb fragment with intermediate affinity for topoisomerase II. **Panel B**: an experiment identical to that shown in panel A except that DNA samples were treated with 20 $\mu$m chromomycin. Lane numbering is as in panel A.

To study this question two identical DNA mixtures were prepared, consisting of equal weights of plasmid pSP64-34, which contains the 657 bp histone SAR, and of the pSP64 vector. Radioactively labeled pSP64-34 or pSP64 probes were added to either one of the two mixtures. Restriction enzyme digestion and end-labeling of each probe were performed so as to yield the SAR at one end of the linear pSP64-34 molecule and the label at the non-SAR end common to both probes (see Materials and methods). The DNA mixtures were incubated with increasing concentrations of topoisomerase II in the presence of VM26, and drug-induced cleavage sites were then determined as described in Materials and methods. Examination of the ethidium bromide-stained gel for this experiment (Figure 5A), shows that for each probe mixture the SAR-containing 3.6 kb fragment was progressively degraded following addition of increasing amounts of topoisomerase II. In contrast, there was no visible reduction in the intensity of the 3.0 kb band corresponding to the vector fragment. Clearly, topoisomerase II preferentially binds and also cleaves the SAR-containing fragment.

The topoisomerase II cleavage products for each probe were then visualized by autoradiography, and are shown in Figure 5B. At a low topoisomerase II:DNA input ratio, we could only detect topoisomerase II cleavage in the 3.6 kb SAR-containing DNA fragment (cf. lanes 2 and 7 for each probe). As expected, major cleavage sites were observed in the SAR region, but strong cleavage sites were also distributed throughout the flanking non-SAR sequences. With addition of more topoisomerase II, the 3.6 kb fragment was increasingly cleaved, and only at topoisomerase II:DNA input ratios corresponding to quantitative precipitation of the SAR probe did the 3.0 kb vector probe start being cleaved (cf. lanes 4 and 5 and 9 and 10). Even then, while most of the SAR-containing 3.6 kb band had been processed into smaller cleavage products, the 3.0 kb vector band remained largely intact.

We carried out an identical experiment without VM26. In the absence of the drug the total yield of cleavage products is strongly reduced but the preferential cleavage in the SAR-containing fragment relative to the control DNA is even more impressive. At low topoisomerase II concentrations cleavage was exclusively observed in the SAR-containing fragment (Figure 5D, lanes 1–4). Only at the highest topoisomerase II concentrations used was cleavage observed in the control DNA (Figure 5D, lanes 11 and 12).

As has been noted before (Udvardy *et al.*, 1986), the patterns of cleavage products in the presence and absence of VM26 are quantitatively very different but qualitatively similar as observed after a longer exposure of the autoradiograms (data not shown). The major cleavage sites observed in the non-SAR region without added VM26 (Figure 5D) are minor ones in the presence of the drug (Figure 5B). Conversely, major cleavage sites observed in the presence of the drug become minor sites in the absence of VM26. The same observation applies for the SAR region as well: while cleavage in the SAR is prominent in the presence of the drug, it is much reduced in its absence. The sequence-dependent cleavage by topoisomerase II is clearly altered by the drug.

In conclusion, SARs act as Circean DNA elements which strongly promote topoisomerase II-induced cleavage in flanking sequences. Although the cleavage pattern is quantitatively strongly affected by VM26, the *Circe* effect of SARs is independent of the addition of this drug. In the absence of the drug, the histone SAR can promote cleavage in the flanking non-SAR DNA in *cis* without containing itself a major cleavage site. In addition, although the presence of a *cis*-acting SAR is clearly required for preferential cleavage, the localization of double-strand breaks is identical in the non-SAR region common to the SAR-containing and control DNA fragments.

### *Inhibition of SAR-specific topoisomerase II binding by distamycin*

Our results show that SARs nucleate in *cis* preferential topoisomerase II assembly and cleavage. The same SARs also nucleate preferential histone H1 assembly; this selective interaction involves the oligo(dA)·oligo(dT) sequences common to all SARs since dA·dT sequence polymers behave as highly efficient artificial SARs (Izaurralde *et al.*, 1989). In addition, we have shown that distamycin A, a drug belonging to the family of bisbenzimidazoles which bind selectively to AT-rich duplex DNA (Martin and Holmes, 1983) could prevent the formation and even dissociate the preferential H1–SAR complexes (Käs *et al.*, 1989).

In the following we establish that the selective interaction of topoisomerase II with SARs also involves (dA)·(dT) tracts. We first examined the effect of distamycin A; preincubation of the DNA probe with 20 $\mu$M distamycin completely inhibited the selective binding of topoisomerase II to the 1.3 kb histone SAR fragment (Figure 6A). Under these conditions, the 4.4 kb pBR322 fragment which is the largest one in the probe precipitated most efficiently. At the concentration used, distamycin A binds selectively to dA·dT tracts in the histone SAR, as shown by DNase I footprinting (Käs *et al.*, 1989). A control experiment shown in Figure

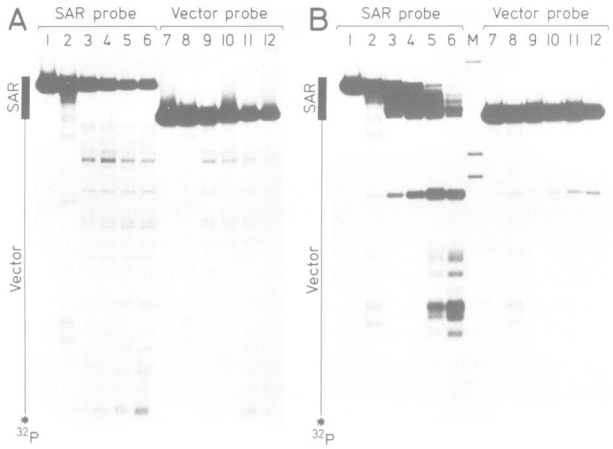


**Fig. 7.** Modulation of topoisomerase II cleavage by distamycin and chromomycin. DNA samples identical to those used in the experiment shown in Figure 5 were treated with 20 mM distamycin or chromomycin prior to incubation with topoisomerase II and VM26-induced cleavage. Reaction conditions were otherwise identical to those given in the legend to Figure 5 (see also Materials and methods). **Panel A**: samples treated with distamycin were incubated with 0 (**lanes 1** and **7** for the SAR or Vector probes respectively), and 50, 100, 150 or 200 ng of topoisomerase II (**lanes 3–6** and **9–12** for the SAR and Vector probes respectively). **Lane 2** (SAR probe) and **lane 8** (Vector probe) are no-drug controls incubated with 50 or 250 ng of topoisomerase II respectively. Note that the cleavage pattern is altered by distamycin. **Panel B**: the experiment is identical to that shown in panel A except that chromomycin was used instead of distamycin in the corresponding samples. Lane numbering is as in panel A. The lane labeled 'M' contained mol. wt standards. Note that the cleavage pattern is altered by chromomycin.

6B, using the drug chromomycin which selectively binds the minor groove of GC-rich DNA (Van Dyke and Dervan, 1983; Fox and Howarth, 1985; Gao and Patel, 1989). This drug did not interfere with the selective precipitation of the 1.3 kb histone SAR by topoisomerase II, and in fact enhanced the selective aggregation of the SAR-containing fragment.

We also examined the effect of these drugs on topoisomerase II cleavage. In an experiment similar to that shown in Figure 5, we analyzed the pattern of VM26-induced cleavage sites using DNA preincubated with 20 $\mu$M distamycin (Figure 7A) or chromomycin (Figure 7B). Whereas the SAR-containing probe was more readily cleaved than the vector probe (cf. lanes 2 and 8 in panel A and see Figure 5) in the absence of drug, cleavage in the SAR and vector probes now occurred with essentially similar efficiencies in the presence of distamycin (cf. lanes 3–6 and 9–12 in panel A). Distamycin thus prevents preferential cleavage of the SAR-containing DNA relative to the control DNA fragment. Closer inspection reveals that distamycin alters the topoisomerase II cleavage pattern (cf. lanes 2 and 3 and 8 and 9 in panel A). Particularly striking is the suppression of cleavage in the SAR region by the drug.

We also analyzed topoisomerase II cleavage of the SAR-containing fragment in the presence of chromomycin. This drug did not inhibit the highly preferential cleavage of the SAR-containing probe relative to the vector probe but had a dramatic effect on the distribution of cleavage sites. In particular, addition of chromomycin resulted in enhancement of cleavage within the SAR region and suppression of several cleavage sites in the vector region (cf. lanes 2 and 3 and 8 and 9 in Figure 7B).

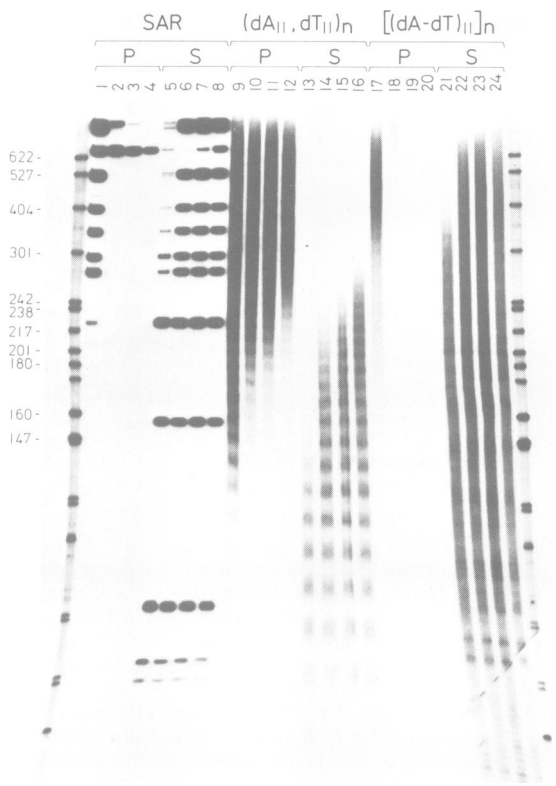


**Fig. 8.** Precipitation of artificial SARs. The end-labeled checkered homopolymer $(dA_{11},dT_{11})_n$ and the alternating polymer $[(dA\text{-}dT)_{11}]_n$ (Izaurralde *et al.*, 1989) were used as probes. The procedure used is essentially the same as that described in the legend to Figure 1. The DNAs in the pellet (P) and supernatant (S) fractions from all samples were purified and run on a denaturing 6% acrylamide/7 M urea gel. **Lanes 1, 5, 9, 13, 17** and **21** are samples incubated with 700 ng of topoisomerase II in the absence of competitor DNA. All the other samples contained 500 ng of salmon sperm DNA. **Lanes 2, 6, 10, 14, 18** and **22** contained 1000 ng of topoisomerase II. **Lanes 3, 7, 11, 15, 19** and **23** contained 700 ng of topoisomerase II. **Lanes 4, 8, 12, 16, 20** and **24** contained 400 ng of topoisomerase II. **Lanes 1–8** show a control experiment using the histone SAR probe, pUL402, which was digested and labeled as described in Figure 1. The SAR is located on the 657 bp fragment which is selectively precipitated (**lanes 3** and **4**).

In summary, distamycin but not chromomycin abolishes the *Circe* effect of SARs as determined both by selective aggregation and cleavage. But both drugs affect the cleavage site distribution; supposedly, binding or cleavage sites containing dA·dT or dG·dC base pairs are negatively interfered with by distamycin or chromomycin respectively.

### *Artificial SARs*

The suppression of preferential topoisomerase II binding to SARs by distamycin strongly suggests that some feature of the AT-richness of SARs plays an important role in specific protein recognition. We tested several synthetic simple-sequence polymers in topoisomerase II aggregation assays to examine this possibility directly, as previously reported for binding to scaffolds and purified histone H1 (Izaurralde *et al.*, 1989). Results are shown in Figure 8 for two of these polymers. Oligo$(dA)_{11}$·oligo$(dT)_{11}$ is composed of runs of 11 As and 11 Ts in an order randomized by ligation, yielding what we refer to as the 'checkered' homopolymer. When tested in the presence of competitor DNA, checkered

polymers above a critical size of ~240 bp were efficiently and selectively precipitated by topoisomerase II (see Figure 8, lanes 10–12 and 14–16, for the pellet and supernatant fractions, respectively). In contrast, the alternating poly(dA–dT) polymer was very poorly bound (lanes 18–20 and 22–24). Figure 8 also shows a control experiment performed with a probe containing the 657 bp histone SAR. At the topoisomerase:DNA input ratios of 0.8 and 1.4 (w/w) shown in lanes 4 and 3 respectively, no fragments other than the 657 bp SAR were recovered in the pellet fraction. This result demonstrates that it is the presence of homopolymeric oligo(dA)·oligo(dT) tracts of sufficient critical size, rather than AT-richness *per se*, which is required to create highly efficient artificial SARs.

Other polymers tested in these studies were poly(dG-dC), poly(dA-dG) and poly(dA-dC). As was the case in our studies with histone H1 (Izaurralde *et al.*, 1989), these simple polymers could not serve as efficient artificial SARs with the possible exception of poly(dA-dC). Above a length of 1.7 kb, corresponding to a critical size seven times larger than that required for poly($dA_{11}\cdot dT_{11}$), this polymer appeared to generate specific complexes (data not shown).

## Discussion

Topoisomerases play pivotal enzymatic roles in cells by solving the topological problems associated with DNA metabolism, including replication, transcription, recombination and chromosome segregation; these enzymes act by nicking transiently either one (type I) or both strands (type II) of the DNA helix (reviewed by Wang, 1985). In this paper we have addressed the question of the structural role of topoisomerase II, the major non-histone component of the metaphase scaffold, which has been shown to be localized at the base of chromatin loops (Earnshaw and Heck, 1985; Gasser *et al.*, 1986). SARs are the DNA elements thought to define the bases of these loops as they interact specifically with the metaphase and nuclear scaffolds (reviewed by Gasser and Laemmli, 1987).

The experiments presented here demonstrate that highly purified topoisomerase II preferentially binds and aggregates SAR-containing DNA. Although clear differences exist in the relative affinities of individual SARs for topoisomerase II, this interaction is remarkably specific; near the optimal protein:DNA ratio and in the presence of salmon sperm competitor DNA, SAR-containing fragments are nearly quantitatively aggregated into a pellet fraction; conversely, non-SAR fragments are nearly quantitatively recovered in the supernatant. We have also carried out similar experiments using filter binding rather than centrifugation to follow the protein–DNA complexes and observed quantitatively identical results with both methods (data not shown). Therefore, most of the DNA–topoisomerase II complexes detected by filter binding form aggregates which can be collected by centrifugation.

The ability of topoisomerase II to bind preferentially SAR-containing DNA has been suggested by previous reports. Sander *et al.* (1987) reported preferential binding of purified *Drosophila* DNA topoisomerase II to the heat shock *hsp70* intergenic DNA where a SAR is located (Mirkovitch *et al.*, 1984) under conditions of competition with the *hsp70* gene fragments. They also observed the presence of several topoisomerase II binding sites on these DNA fragments which required high molar ratios of protein to DNA to achieve binding saturation. It has been reported that at such high molar topoisomerase II:DNA ratios, the enzyme catalyzes the catenation of DNA rings (Hsieh, 1983). Because the catenation reaction is known to be dependent on DNA aggregation (Krasnow and Cozzarelli, 1982), topoisomerase II itself could promote DNA aggregation. We found that this actually occurs under our experimental conditions. While we do not know at present how soluble topoisomerase II aggregates DNA, this precipitation phenomenon suggests the presence of DNA-dependent protein–protein interactions.

We show in this study that the interaction of DNA with topoisomerase II is highly cooperative. In the presence of DNA, all the available topoisomerase II (up to a certain concentration) is DNA-bound, forming a complex which can be collected by centrifugation (Figure 3). If the input concentration exceeds one topoisomerase II dimer per 200 bp of DNA, soluble enzyme is then recovered in the supernatant (Figure 3A). This value is close to that reported for the interaction of DNA with bacterial DNA gyrase, a protein which shares extensive amino acid homology with eukaryotic type II topoisomerases (Wyckoff *et al.*, 1989), and interacts with ~140 bp of DNA (Liu and Wang, 1978; Kirchhausen *et al.*, 1985).

The exact distribution of topoisomerase II along the DNA is not known but the high linear density of about one dimer per 200 bp and the large size of this dimer (300 kd) suggest a continuous distribution along the SAR and flanking non-SAR sequences. Indeed, studies using exonuclease *Bal*31 demonstrate that both the SAR and the non-SAR DNA ends are protected from digestion under conditions of specific complex formation (Figure 4).

The relative binding affinity of a given SAR increases with the length of the flanking DNA (Figure 1B); while a protein:DNA ratio of 1.5 (w/w) is required to precipitate quantitatively and specifically the 657 bp histone SAR, it is possible to lower the protein:DNA ratio by a factor of ~2 if this same SAR is flanked at one end by the 3 kb pSP64 vector. This observation is consistent with the enzyme being distributed along the SAR and its flanking DNA; the favorable free energy gain of topoisomerase II binding in the SAR region appears to be cumulative and to be propagated into flanking sequences since increasing the length of the non-SAR DNA raises the relative binding affinity.

Results of our cleavage experiments are particularly compelling in that respect. These experiments, carried out as a function of the topoisomerase II concentration, demonstrate the *Circe* effect of SARs; a SAR-containing DNA fragment is, at limiting enzyme concentrations, exclusively cleaved by this enzyme as compared with a non-SAR fragment (Figure 5A). This cleavage reaction is by no means restricted to the SAR region but occurs at discrete sites throughout the flanking DNA (Figure 5B). The cytotoxic drug VM26 used in some of these experiments, enhances the extent of the cleavage reaction and strongly affects the cleavage pattern but the *Circe* effect is, if anything, more pronounced without the drug. The effect of VM26 on the cleavage pattern illustrates the potential problem of using this drug to map topoisomerase II cleavage sites *in vivo* or to determine a cleavage consensus (Sander and Hsieh, 1985; Rowe *et al.*, 1986; Udvardy *et al.*, 1986).

The histone SAR can selectively bind topoisomerase II and

exert a *Circe* effect without containing major cleavage sites itself. Major cleavage sites were detected in the histone SAR only in the presence of VM26, while if the drug was omitted, cleavage occurred predominantly in the flanking non-SAR DNA. Thus, sequence-dependent binding and cleavage do not necessarily colocalize. Not all SARs behave similarly: the intergenic *hps70* SAR contains strong selective cleavage sites independent of the use of VM26 (Udvardy *et al.*, 1985, 1986; E.Käs and U.K.Laemmli, unpublished results). Although we show topoisomerase II DNA binding to be cooperative, cleavage sites are clearly not localized at every binding site. The obvious differences in the rate of topoisomerase II cleavage at different binding sites suggest that certain DNA sequences determine the observed cleavage specificity of the enzyme.

The specific interaction of SARs with the nuclear scaffold and histone H1 is due to the presence of several oligo(dA)·oligo(dT) tracts as determined in experiments using synthetic polymers and the drug distamycin (Izaurralde *et al.*, 1989; Käs *et al.*, 1989). The results are similar with topoisomerase II. The specific aggregation and cleavage reaction of SAR-containing DNA by topoisomerase II is abolished in the presence of distamycin, a DNA-binding ligand with a high specificity for the minor groove of dA·dT sequences (Van Dyke *et al.*, 1982; Fox and Waring, 1984). We have used the drug chromomycin as a control, which specifically binds the minor groove of G·C base pairs (Gao and Patel, 1989). The presence of chromomycin had no effect on the specific SAR binding to topoisomerase II as it also did not interfere with the selective interactions of SARs with either nuclear scaffolds or histone H1 (Käs *et al.*, 1989).

The DNA minor groove-binding agents distamycin and chromomycin also alter the topoisomerase II cleavage pattern. Presumably, cleavage sites containing dA·dT or dG·dC base pairs are suppressed by distamycin or chromomycin respectively. Addition of chromomycin resulted in enhancement of cleavage within the SAR region and suppression of several cleavage sites in the vector region. In contrast, addition of distamycin had principally a converse effect. It might be of interest to study the possible cytotoxic synergism of distamycin and/or chromomycin used in concert with drugs like VM26.

Our experiments with simple DNA polymers clearly implicate oligo(dA)·oligo(dT) sequences in preferential topoisomerase II assembly. The 'checkered' $(dA_{11}\cdot dT_{11})_n$ homopolymer interacts highly selectively with topoisomerase II above a critical length of ~240 bp. The critical length determined for histone H1 with the same polymer is shorter (~140 bp). No other polymers we have tested, including the equally AT-rich alternating poly(dA-dT) duplexes showed comparably efficient and selective aggregation by topoisomerase II. We conclude that oligo(dA)·oligo(dT) tracts and not AT-richness *per se* are required to create efficient artificial SARs.

The nature of the selective interaction of topoisomerase II with SAR-containing DNA is very similar to that described for histone H1. Both proteins bind cooperatively to DNA and oligo(dA)·oligo(dT) tracts are involved in the specific interaction of SARs with histone H1 and topoisomerase II as well as with the nuclear scaffold. Topoisomerase II is a major component of the nuclear scaffold and a residual amount of histone H1 could also be detected in scaffolds (Gasser *et al.*, 1986; Käs *et al.*, 1989). Although it is reasonable to think that these proteins are involved in mediating the SAR–scaffold interaction, the experiments presented here do not establish this notion conclusively.

According to the twin supercoiled domain model, transcription of DNA will generate two supercoiled domains of opposite polarities ahead of and behind the moving transcription complex (Liu and Wang, 1987). Recent studies in yeast have demonstrated the validity of this model (Brill and Sternglanz, 1988; Giaever and Wang, 1988). SARs, which flank genes and are particularly close to strong promoters, are thought to tether the chromatin fiber to the nuclear scaffold and consequently define a loop which should experience transcription-dependent supercoiling; in a highly active loop, relaxation might conceivably be rate limiting in the absence of a facilitating mechanism. SARs flanking such a domain might serve this purpose by exerting a *Circe* effect, luring topoisomerase II to the loop to permit rapid relaxation and high levels of transcription. In eukaryotes, both topoisomerase I and II can relax either positively or negatively supercoiled DNA. Recently we have characterized another protein showing preferential SAR-binding properties. Likely to be more than a mere coincidence, this protein was identified as topoisomerase I (J.Falquet and U.K.Laemmli, to be published).

Topoisomerase II is the major non-histone protein of metaphase scaffolds and the highly cooperative, selective association of topoisomerase II with SARs clearly support a chromosome model in which topoisomerase II plays a structural role, fastening DNA loops to the scaffold via specific interactions with SAR sequences. It is easy to see how an orderly aggregation of the SARs mediated by topoisomerase II could tether the chromatin fiber together thus leading to chromosome condensation. However, the experiments reported here and those concerning the preferential association of histone H1 were carried out with naked DNA (Izaurralde *et al.*, 1989; Käs *et al.*, 1989). It will be necessary to study these phenomena in the living cell.

## Materials and methods

### *Yeast strains, media and drugs*

The *S.pombe* strain HM123 (*h⁻leu1*) harboring plasmid pDB(top2)-1 containing the topoisomerase II gene (Uemura and Yanagida, 1986) was grown in YEA (complete rich medium: 3% glucose, 0.5% yeast extract, to which 1.7% agar was added for plates) or EMM2 minimal medium (Mitchison, 1970), to which 2% agar was added for plates. Distamycin A (Sigma) and Chromomycin $A_3$ (Boehringer) was stored as 1 mM stocks in 96% ethanol and absolute ethanol respectively. VM26 (epipodophyllotoxin) was obtained from Bristol-Myers, and stored as a 2.5 mM stock solution in dimethylsulfoxide.

### *DNA procedures*

DNA manipulations were all according to standard laboratory procedures (Maniatis *et al.*, 1982) and knotted phage P4 DNA was prepared as described by Liu and Davis (1981). Artificial DNA polymers were prepared as described previously (Izaurralde *et al.*, 1989).

### *Purification of topoisomerase II from S.pombe*

*Schizosaccharomyces pombe* strain HM123 harboring the *top2* gene on a multicopy plasmid was used as source of topoisomerase II. Ten liters of YEA medium were inoculated with 1 l of a saturated ($3 \times 10^7$ cells/ml) culture grown in EMM2 medium. Cells were grown overnight with aeration at 33°C. The late log phase cells ($2-3 \times 10^7$ cells/ml) were harvested by filtration through a glass filter (Whatman GF/B) and washed once with purification buffer TEG (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 10% glycerol, 1 mM 2-mercaptoethanol). A wet pellet of ~50 g was obtained after centrifugation at 2000 *g* for 5 min.

All steps following harvesting were carried out at 4°C. The cell pellet

was resuspended in 80 ml TEG supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 80 ml of glass beads (0.5 mm in diameter) were added to the suspension. The mixture was placed in Erlenmeyer flasks and vigorously agitated in an ice-water bath (New Brunswick shaker G76). The lysate was recovered by centrifugation at 2500 *g* for 10 min to remove debris. The supernatant (fraction 1) was then centrifuged at 50 000 *g* for 15 min. The resulting pellet was resuspended in 80 ml of TEG supplemented with 100 mM NaCl and 1 mM PMSF. After rocking for 30 min, the suspension was recentrifuged at 50 000 *g* for 15 min. The pellet was then resuspended in 80 ml of TEG supplemented with 400 mM NaCl and 1 mM PMSF. This suspension was rocked for an additional 30 min and again centrifuged at 100 000 *g* for 60 min (Uemura *et al.*, 1987).

The supernatant (fraction II) was then diluted with TEG supplemented with 1 mM PMSF to adjust the final salt concentration to 250 mM NaCl and applied to a 16 ml phosphocellulose (Whatman P-11) column (1.6 × 8 cm) equilibrated with TEG supplemented with 250 mM NaCl and 0.5 mM PMSF. The column was washed with 8 column volumes of the same buffer and proteins were eluted with a 160 ml linear gradient of 250–700 mM NaCl (final concentrations) in TEG and 0.5 mM PMSF. The fractions containing the topoisomerase II activity (eluted at ~600 mM) were pooled (fraction III) and diluted 2-fold with TEG supplemented with 0.1 mM PMSF. The diluted fraction III was loaded onto a 4 ml heparin-Ultrogel column (IBF-A4R; 1 × 5 cm) equilibrated with TEG plus 300 mM NaCl and 0.1 mM PMSF. The column was eluted with a linear gradient of 300–750 mM NaCl (final concentrations) in TEG plus 0.1 mM PMSF. The active fractions were pooled (fraction IV) and diluted 2-fold with TEG plus 0.1 mM PMSF. The diluted fraction IV was applied to a Mono-Q column (Pharmacia Fine Chemicals; 0.5 × 5 cm) equilibrated with 20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM 2-mercaptoethanol, 10% glycerol, 0.1 mM PMSF (TEG-Q buffer), plus 250 mM NaCl. After washing the column with the same buffer, proteins were eluted with a 20 ml linear gradient of 250–500 mM NaCl (final) in TEG-Q. Each active fraction (0.5 ml) was diluted 2-fold with 87% glycerol in TEG-Q and then stored at −20°C. Only the purest fraction (examined by SDS–PAGE followed by silver-staining) was used in these experiments. This fraction (1 ml contained 500 μg of topoisomerase II whose sp. act. (defined below) was $1.1 \times 10^6$ U/mg protein.

We obtained a nearly homogeneous preparation of topoisomerase II as judged by SDS–PAGE. Although some minor bands were observed when the gel was stained with silver nitrate, no bands other than the 150 kd topoisomerase II could be observed in the gel stained with Coomassie blue when 5 μg of the protein was loaded. No ATP- and $Mg^{2+}$-independent (topoisomerase II) relaxation activity could be detected in this fraction (data not shown). No degradation of the protein was detected during the period of this work (4 months); however, we observed a small loss of activity.

### *Assays of topoisomerase II activity*

Topoisomerase II activity was assayed throughout the purification by measuring its unknotting activity using P4 phage DNA as a substrate (Liu and Davis, 1981). Reaction mixtures (20 μl) contained 10 mM Tris–HCl, pH 7.9, 50 mM KCl, 100 mM NaCl, 10 mM $MgCl_2$, 50 μg/ml gelatin, 1 mM ATP and 0.1 μg of knotted phage P4 DNA. Topoisomerase II (freshly diluted in 10 mM Tris–HCl, pH 7.5, 10 mM 2-mercaptoethanol, and 1% CHAPS) was added, samples were incubated at 33°C for 15 min, and the reaction was stopped by addition of 5 μl of 5% SDS, 50 mM EDTA, 30% glycerol, and 0.1% bromophenol blue. The samples were run on 0.8% agarose gels in Tris–acetate running buffer. One unit of the enzyme is defined as the amount required to convert half of the input knotted P4 DNA into a nicked circular form under these reaction conditions.

### *Filter binding and precipitation assays*

Filter binding and precipitation assays were performed as described (Izaurralde *et al.*, 1989). Most experiments were carried out in buffer TEN (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.1% digitonin, 100 U/ml Trasylol (Bayer) and 25 μg/ml bovine serum albumin). Some experiments were performed in buffer B (see below), and yielded similar results. The topoisomerase II stock (500 ng/μl) was appropriately diluted in TEN and added to the reaction mixture containing DNA (50 μl final volume). The DNA mixture consisted of 0.5 μg sonicated salmon sperm and 1–2 ng of the end-labeled probe being tested. After a 30-min incubation at 30°C, the samples were spun down in a microfuge (10 min at 4°C). The fractions of precipitated end-labeled probes were determined by Cerenkov counting. Pellets were washed once with TEN and resuspended in 50 μl of the same buffer and 2.5 μl each of 10% SDS and 10 mg/ml Proteinase K were added to the pellet and supernatant fractions. The samples were incubated for 60 min at 60°C. In some experiments, the salmon sperm competitor DNA was substituted with 200 ng of an equal weight mixture of *Eco*RI-linearized plasmids pSP64 and pSP64-34 (see Results).

The exonuclease *Bal*31 digestion experiment shown in Figure 4 was performed in buffer B (20 mM Tris–HCl, pH 7.4, 20 mM KCl, 70 mM NaCl, 0.05 mM spermine, 0.125 mM spermidine, 0.1% digitonin, 100 units/ml Trasylol, 25 μg/ml BSA, 1 mM dithiothreitol. The end-labeled histone probe was mixed with 500 ng of salmon sperm DNA in 100 μl of buffer B and incubated with increasing amounts of topoisomerase II as indicated in the figure legend. After 30 min at 30°C, samples were divided into two aliquots and $CaCl_2$ was added to 12 mM. In one set, 0.1 units of *Bal*31 were added to each sample; digestions were terminated after 20 min at room temperature by addition of 20mM EGTA. Samples in the other set were spun down. The pellet and supernatant fractions and the *Bal*31-digested samples were loaded on an agarose gel following treatment with proteinase K.

For the experiment shown in Figure 8, the pellet and supernatant DNA fractions were first purified by phenol extraction and ethanol precipitation before electrophoresis on a 6% acrylamide–7 M urea denaturing gel.

### *Drug interference experiments*

DNA mixtures similar to those described above were preincubated with drugs as follows. The DNA (probe plus competitor) was mixed with an equal volume of ×2-concentrated drug freshly diluted in 10 mM Tris–HCl, pH 7.4, 15 mM NaCl (also containing 0.1 mM $MgCl_2$ for chromomycin). The final DNA concentration was always kept at 100 μg/ml to maintain the ratio of base pairs per molecule of drug constant. Aliquots containing the standard amount of competitor DNA and probe were then mixed with topoisomerase II in a 50 μl volume (final) of TEN buffer, and samples were treated as described above.

### *Topoisomerase II cleavage reactions*

Reaction mixtures identical to those described above were analyzed for topoisomerase II-mediated cleavage as follows. A 50 μl (final volume) reaction containing DNA (probe plus competitor) in buffer B and increasing amounts of topoisomerase II (as indicated in the figure legends) was equilibrated for 5 min at 30°C. ATP was then added to 1 mM and VM26 to 50 μM (final concentration) and incubation allowed to continue for an additional 5 min. Reactions were stopped by addition of 1% SDS, followed by 20 mM EDTA and 250 μg/ml proteinase K. Samples were then incubated for 2–3 h at 60°C, the DNAs were purified by phenol extraction and ethanol precipitation and electrophoresed on 1% agarose gels.

DNA mixtures consisting of cold *Eco*RI-linearized plasmids pSP64 and pSP64-34 added as carriers, mixed with ~$10^6$ c.p.m. of either probe, uniquely end-labeled with $^{32}P$. 125 ng of each plasmid (carrier plus probe) were present in each sample. Uniquely end-labeled probes were prepared by digestion vector pSP64 and clone pSP64-34 (containing the 657 bp histone SAR cloned in the *Sma*I site of pSP64) with *Hin*dIII, followed by end labeling using the Klenow fragment of *Escherichia coli* DNA polymerase I. After purification, the plasmids were redigested with *Bam*HI, yielding molecules labeled at their common (SAR-distal) ends, while the other labeled end was recovered on a short *Hin*dIII–*Bam*HI polylinker fragment. In some experiments, the plasmids were labeled at the *Bam*HI (SAR-proximal) end and recut with *Hin*dIII to analyze cleavage within the SAR region more precisely (data not shown).

### *SDS–PAGE analysis of protein fractions*

SDS–PAGE of protein was carried out according to Laemmli (1970). Gels were stained with Coomassie blue or with silver nitrate. Protein concentration was estimated by the method of Bradford (1976) or by scanning the SDS–polyacrylamide gel stained with Coomassie blue. BSA (Sigma) was used as a standard.

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