Binding Specificity of a Nuclear Scaffold: Supercoiled, Single-Stranded, and Scaffold-Attached-Region DNA‡

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ABSTRACT: Scaffold-attached-region (SAR) elements of DNA enhance transcriptional rates, and this has been correlated with their ability to undergo separation into single strands (ssDNA) under conditions of negative superhelicity (Bode et al., 1992). The competition studies presented here suggest that the SAR–scaffold interaction is based, in part, on the recognition of single strands, while about one-half of SAR sites are inaccessible to ssDNA. Conversely, since there are 20 000 SAR sites but more than 60 000 sites for ssDNA per nuclear equivalent, not all ssDNA sites are open for SARs. In addition, a completely separate set of binding centers recognizing and enzymatically converting DNA of superhelical density below –0.04 can be titrated. These findings reflect multiple binding specificities for scaffold preparations that are routinely used for screening scaffold-attached regions.

Within eukaryotic nuclei, the genome is organized into topologically constrained, independently regulated looped domains (Mirkovitch et al., 1987). Domains, which are either transcriptionally active or poised for transcription, show an elevated level of DNAse sensitivity that decreases at their borders (Bonifer et al., 1990, 1991; Dillon & Grosveld, 1993; Lawson et al., 1982; Levy-Wilson & Fortier, 1989). Typically, these borders coincide with regions that are associated with the nuclear skeleton [S/MAR[1] -elements; cf. Fritton et al. (1988), Jarman and Higgs (1988), Levy-Wilson and Fortier (1989), and Phi-Van and Strätling (1988)].

The proteinaceous backbone of the eukaryotic nucleus can be isolated and characterized according to various protocols optimized for the removal of soluble nuclear components. Depending on the agent used for extraction (high salt or LIS), the resulting structure has been termed either "nuclear matrix" or "nuclear scaffold" (Mirkovich et al., 1984). Hence, DNAs with an affinity for these entities were called either MARs (Cockerill & Garrard, 1986) or SARs (Gasser & Laemmli, 1986), although more recent research has shown that these elements are indistinguishable.

The preparations of the nuclear scaffold investigated here have been utilized extensively for the discovery and characterization of scaffold-attached regions, i.e., SAR sequences. During these studies, we established a general correlation between the affinity of the scaffold–SAR interaction and their influence *in vivo* on transcriptional rates (Bode et al., 1992; Klehr et al., 1991, 1992; Mielke et al., 1990). Contrary to the classical enhancers, SAR elements exert their effects only for transgenes that have assumed their final chromatin structure after integration into the genome of the host cell (Klehr et al., 1991; Stief et al., 1989). Two SAR elements that flank a gene, forming a "minidomain", were found to act in a synergistic manner (Klehr et al., 1992; Schlake et al., unpublished results).

Further evidence for the participation of the nuclear skeleton in gene expression comes from the preferential association of actively transcribed genes, the localization of RNA synthesis and pre-mRNA splicing, and the presence of some rare, tissue-specific, promoter-binding factors (Bidwell et al., 1993; van Driel et al., 1991; Stuurman (1992) and references therein). It is obvious that the proteins constituting the skeleton must have diverse recognition modes for DNA structures or sequences. Besides some rare transcription factors, there are groups of constitutive DNA-binding proteins such as topoisomerases and histone H1 (Izzauralde et al., 1989; Käs et al., 1989), components of the internal fibrogranular network [the "matrins", cf. Berezney (1991), Hakes and Berezney (1991), and Nakayasu and Berezney (1991)], and at least one component of the nuclear lamina [lamin B1, cf. Ludérus et al. 1992)].

In the present article, binding sites for DNA structures with a role in transcription, i.e., superhelical (Dunaway & Ostrander, 1993; Jupe et al., 1993; Tsutsui et al., 1988) and single-stranded forms of DNA (Bode et al., 1986; Probst & Herzog, 1985; Palecek et al., 1993) as well as linear, double-stranded SARs, are quantified and their interrelationship is investigated.

MATERIALS AND METHODS

Plasmids. A 2.2-kb *Eco*RI fragment (SAR_{2200}) and its 0.83-kb subfragment (SAR_{800}), derived from the human INF-β upstream SAR element, were each cloned into the polylinker of plasmid pTZ18R (Bode & Maass, 1988; Mielke et al., 1990).

Cell Culture and Isolation of Nuclei. The procedures for culturing mouse-L-2_4 cells and the isolation of nuclei were as described previously (Bode et al., 1986; Bode & Maass, 1988).

Nuclear Scaffolds. Nuclei were subjected to the LIS extraction procedure of Mirkovitch et al. (1984) to obtain nuclear halos; details of our precise protocol have been published previously (Mielke et al., 1990). The degradation of the resulting halos with an appropriate set of restriction endonucleases depended on the type of experiment and the

‡ This contribution is dedicated to Prof. Karl Wagner on the occasion of his 65th birthday.

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[1] Abbreviations: SAR, scaffold-attached region; MAR, matrix-attached region; S/MAR, consensus term covering SARs and MARs; IFN, interferon; huIFN-β, human interferon-β; LIS, 3,5-diiodosalicylic acid.

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type of DNA used. Usually, halos were degraded with *Bgl*II to obtain the scaffolds; see the figure legends for details.

Binding Experiments. The scaffolds were adjusted to 700 μL with digestion buffer. They were partitioned into seven aliquots (7×10^6 cell equiv of nuclear scaffolds each). Unless stated otherwise, they were provided with an excess of an unspecific competitor (*Escherichia coli* genomic DNA suspended in the same buffer) to a final concentration of 0.66 mg/mL.

(a) Standard Assays. The vials were provided either with linear restriction fragments that had been end-labeled by Klenow polymerase using [α-^{32}P]dATP or with unlabeled, covalently closed plasmids of different topology which had been prepared by the action of topoisomerase I in the presence of various amounts of ethidium bromide. The preparation and subsequent identification of topoisomers closely followed the procedure of Keller (1975). They were adjusted to 150 μL with digestion buffer and incubated on a rocking mixer at 37 °C overnight. After the usual separation into pellet (P) and supernate (S) fractions followed by DNA purification (Mielke et al., 1990), aliquots of P and S dissolved in 100 μL of TE were separated on 1% agarose gels. DNA was then blotted onto a nylon screen (Bio-Rad Zeta probe). Depending on the problem, the visualization of species followed different routes: (i) In cases where a Klenow reaction had been used for radioactive end-labeling of distinct fragments, 1000–5000 cpm equiv per trace could be detected directly by autoradiography overnight (Agfa Curix RP1 or Kodak X-Omat AR; Figures 3A, 4B, and 7A). Where applicable, the same blots could be used for a subsequent hybridization with a nick-translated, radioactive probe of pCL to visualize competitor DNAs (Figures 3B, 4A,C, and 7B). (ii) Supercoiled plasmids were always visualized by hybridization with labeled pCL (cf. Figures 1 and 2).

(b) Competition Assays. Each vial was incubated with an equal amount of DNA (either radioactively end-labeled restriction fragments or supercoiled plasmids). Then, an increasing molar excess of competitor (supercoiled plasmids or heat-denatured single-stranded DNA with a hyperchromicity of at least 30%, respectively) was added as described in the legends to Figures 3 and 4. All samples were processed as described in Standard Assays. After the respective DNA pellets were dissolved in 50 μL of TE, aliquots were applied to a 1% agarose gel. Where applicable, the appropriate fractions were deduced from the volumes of a control prepared from a SAR and a vector fragment required to yield equal counts for P and S (see the Results section). The gel was blotted as described above and exposed to an X-ray film before and after hybridization with a nick-translated probe of pCL.

(c) Kinetics. For kinetic measurements, a total, nonaliquoted preparation of 700 μL was used in which plasmids pTZ and pCL were incubated analogously to the standard assays, but taking into account the larger volume. At the appropriate times, aliqots were withdrawn and immediately subjected to the usual DNA purification protocol with or without separating the aliquots into P or S fractions beforehand (cf. Figure 1B,C, respectively).

Sequential LIS Extraction. A protocol was developed to gradually extract soluble chromatin components of isolated nuclei from one extraction step to the next using increasing LIS concentrations. Nuclei isolated from four plates of 150 cm^2 (1×10^8 cells) were subjected to this procedure. After each extraction in 5 mL of LIS buffer, an aliquot of the homogenate was withdrawn. This aliquot and the remainder were both pelleted (2400*g*, 5 min, 4 °C); the remainder was subjected to the next extraction step, while the aliquot was directly processed in a 10-mL vial using digestion buffer as described (Mielke et al., 1990). Halos obtained from all extraction steps were transferred into 1.5-mL capped tubes, which were filled to 100 μL with digestion buffer and treated with 100 units of *Bgl*II. After the nuclear scaffolds had been homogenized by pipeting, they were diluted to 200 μL with digestion buffer, and 100 μL were used for binding experiments according to the standard assays.

Fitting of Binding Curves. Curve fitting was performed according to the published procedures (Mielke et al. 1990), but used another program, Sigma plot, for calculation and representation.

RESULTS

SAR elements harbor no sequences that might lead to their unambiguous identification. Although they are generally AT-rich and there is a recurrence of certain boxes (Cockerill & Garrard, 1986), several diverse sequences such as AATATATTT or $(GA)_n$ tracts (T. Kohwi-Shigematsu and J. Bode, unpublished results) are compatible with SAR activity *in vitro* and *in vivo*. The recent observations that most functional SAR sequences separate strands under the natural superhelical density of a plasmid (Bode et al., 1992; Kohwi-Shigematsu & Kohwi, 1990) and that the above motifs are nucleation centers for this event led to the present investigation. Although unwinding occurs more readily in circular, torsionally stressed molecules, the binding of linear SAR fragments could depend on such a structural property (Dickinson et al., 1992). Moreover, the single-stranded portions, which would be more prominent in SAR-containing rather than SAR-free plasmids, could conceivably promote the recognition of SAR sequences in this context.

Recognition Functions of Nuclear Scaffold Preparations. Our initial experiments confirm and extend the observation by Tsutsui et al. (1988) that superhelical DNA is retained by scaffold preparations. In Figure 1A we show the binding of plasmid pCL at the original negative superhelical density of a bacterium. pCL, which contains the 800-bp SAR insert including the center of unwinding as identified by Bode et al. (1992), was equilibrated with the scaffold at 37 °C overnight. This time span proved to yield the most reproducible results, as the reassociation and enzymatic conversions are slow processes requiring at least 6–8 h until a stable pattern and/or distribution over the P and S fractions is reached. Analyses performed on equivalent fractions of the pelletable fraction (P) and the supernate (S) show that a substantial part of the plasmid had become bound to the scaffold. Some action of topoisomerase(s) is evident from a faint ladder of topoisomers in fraction S that was not present in the original mixture (T). In addition to the topoisomers, a significant contribution from the nicked form is observed which accumulates in the unbound (S) fraction. We have to conclude that the recognition of the SAR insert is impeded in the covalently closed nicked form and in at least the majority of topoisomers. We will show here that the association of the highly supercoiled forms is also independent of the SAR sequence and that it represents a distinct phenomenon.

Figure 1B compares the on-rates of binding (i.e., occurrence in the P fraction with time) for the SAR-free plasmid pTZ and the analogous SAR plasmid pCL, which are half-maximal around 0.5 h in both cases. Nicked forms of both plasmids are retained first within the scaffold (P). At later times, nicked pTZ appears to be released in preference to pCL (see the 22-h time point). This may be a simple consequence of its smaller

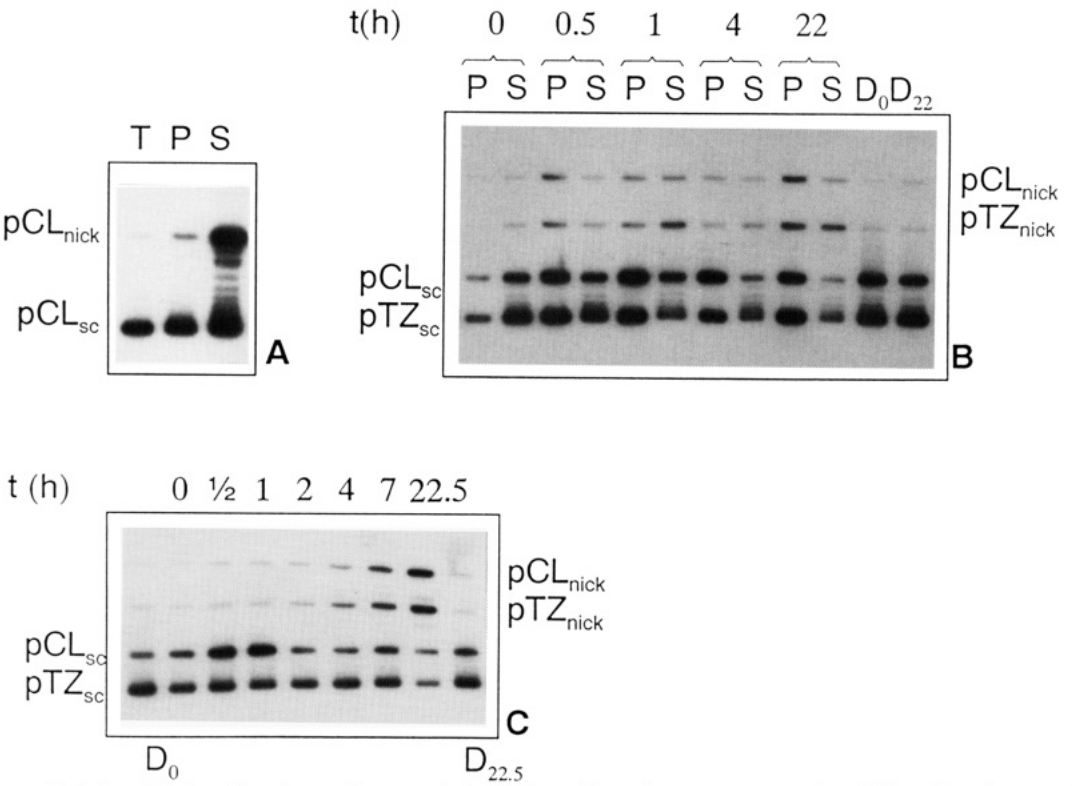

FIGURE 1: Supercoiled plasmids bound to the nuclear matrix but released into the supernatant after nicking. Topoisomers visualized by autoradiogaphy after hybridizing the blots with a nick-translated probe of plasmid pCL. (A) Topoisomers of reduced superhelicity are produced during the incubation of the SAR vector pCL (500 ng) with scaffolds obtained from mouse cells under the conditions of a standard assay (Materials and Methods). After the samples were separated into pellet (P) and supernate (S) fractions, they were purified and 0.1% aliquots of each were analyzed on a 1% agarose gel: T, reference, i.e., the pCL plasmid incubated under the same conditions but without scaffolds; pCL_{nick}, nicked plasmid; pCL_{sc}, supercoiled plasmid. (B) The binding of supercoiled plasmid DNA to the nuclear scaffold occurs independently of an SAR insert: the time-dependent binding and nicking of an equimolar mixture of pTZ and pCL by nuclear scaffolds is shown. Scaffolds obtained from two plates of mouse cells were incubated with 0.75 pmol of pTZ and pCL under standard conditions. Equal aliquots were withdrawn at different times (0–22 h), immediately separated into P and S fractions, and submitted to the usual DNA purification scheme. Equal fractions (0.4%) of either P or S were applied to the gel. Controls containing plasmids but no scaffolds were incubated the same way, and aliquots were taken at the beginning (D_0) and at the end (D_{22}) of the incubation. (C) Kinetics of the nicking reaction. The experiment was performed as described for B but without separation into P and S fractions.

molecular size or it may indicate some residual recognition of the SAR sequence in the relaxed circle.

The experiment in Figure 1C resembles that in Figure 1B, with the exception that the separation into P and S fractions was omitted to enable a more direct comparison of the nicking rates. Since the kinetics are indistinguishable, it is concluded that supercoiled forms of both plasmids are bound and processed irrespective of the presence of an SAR insert.

Recognition of Topoisomers According to Superhelical Densities. Since plasmids are recognized at the bacterial superhelical density but not at the reduced superhelicity of topoisomers produced during the incubation with a scaffold (see Figure 1A and lanes 9 and 10 in Figure 2), we decided to characterize the topoisomers close to the transition from the binding to the nonbinding states. In addition to the mixture of supercoils isolated from bacteria (T_{sc}, Figure 2), we have therefore prepared other sets of topoisomers representing the appropriate range of reduced linking numbers. This was achieved by the action of topoisomerase I in the presence of 10 or 12.5 μmol/L of the intercalator ethidium bromide (T_{10} and $T_{12.5}$ in Figure 2), which stabilizes the underwound state during the nicking–closing reaction (Keller, 1975, Gruskin & Rich, 1993). A standard was provided by first nicking the plasmid with traces of DNAse I followed by religation. The resulting topoisomer (linking number difference $\alpha = 0$) was used to identify and isolate a marker ($\alpha = -2$) from a ladder of topoisomers, which was used for a calibration of Figure 2 (cf. lane 7).

After the usual equilibration of the three sets of topoisomers with the scaffolds, we again applied equivalent fractions of the P and the S fractions to enable a direct comparison of the binding capacity. Figure 2 demonstrates that the transition occurs within a very narrow interval, between topoisomers $\alpha = -16$ (totally bound) and $\alpha = -12$ (totally unbound, cf. lanes 2 and 3), i.e., around superhelical densities of –0.04.

Binding Sites of Supercoiled DNA and Linear SAR DNA Are Unrelated and Nonoverlapping. The relation of sites for binding double-stranded, linear SAR elements and supercoiled plasmids was investigated further by competition assays. For these analyses, we have applied a *modified equal counts* approach, i.e., we have refined the definition of equivalent fractions (the volumes that have to be applied to the P and S traces in a gel): As an additional control, an aliquot of each scaffold preparation was supplied with equal amounts (counts) of nonbinding and SAR fragments (arising from the excision of SAR DNA from an SAR vector followed by simultaneous labeling of both fragments by the Klenow reaction). The equivalent fractions of the pellet and supernates, i.e., the volumes yielding equal counts, could then be determined (cf. the traces marked control in Figures 3 and 4). These sample volumes for P and S were maintained for all samples in a given experiment, irrespective of the presence of competing species. This modification of the standard protocol has the following advantages: (i) a coarse classification of affinities, i.e., species binding more weakly than the standard are automatically driven to the supernate (S) fraction, and (ii) a

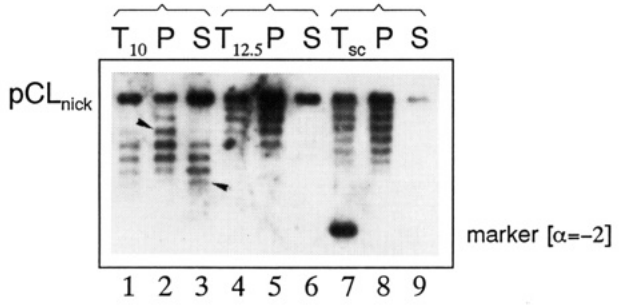

FIGURE 2: Onset of binding topoisomers to the nuclear matrix occuring in a very narrow interval of linking numbers. Topoisomer mixtures (200 ng) of plasmid pCL were incubated with nuclear scaffolds in a standard assay. Equal fractions of P and S (2% each) were applied to an 0.8% agarose gel in the presence of 10 μmol/L chloroquine; the intercalator determines the mode of separation (see below). T_{10} and $T_{12.5}$ designate the input mixtures of the topoisomers obtained at the respective concentrations (μmol/L) of ethidium bromide; T_{sc} indicates the topoisomers as isolated from *E. coli*. A topoisomer of known linking number difference ($\alpha = -2$) was added in lane 7 as a marker. The arrowheads mark topoisomers of different α values: lane 2, −16; lane 3, −12. Regarding the mode of separation, note that while more highly supercoiled molecules are usually more compact and migrate faster (cf. Figure 1A), the order of mobilities is reversed for the present analyses. This is due to the fact that the amount of intercalator used for an optimal resolution causes supercoiling in the opposite direction (Bauer et al., 1980).

sensitive detection of competitors. In the simplest case, the supernate fraction of the control is free of binding DNAs. Addition of a competing specimen leads to the appearance of the standard in the supernate (S) fraction, which is easily monitored due to a lack of background.

As an example, the scaffold investigated in Figure 3 showed exclusive binding of SAR_{800} to the pellet and complete appearance of the vector part (pTZ_{lin}) in the supernate. Six aliquots of this scaffold preparation were then loaded with the same amount of SAR_{800} and increasing amounts (1–100-fold relative to SAR_{800}) of unlabeled supercoiled plasmid (pTZ_{sc}). All samples were incubated at 37 °C as described in Materials and Methods. After the separation into bound (P) and unbound fractions (S), electrophoresis, and blotting, the respective autoradiograph of the blot revealed that SAR_{800} remained unaffected by any excess of supercoiled pTZ (Figure 3A, lanes 1–12). Visualization of pTZ by a complementary probe (nick-translated pCL) showed that sufficient pTZ_{sc} persists during the incubation to saturate its respective sites (Figure 3B; note the appearance of pTZ_{sc} in the S fraction starting with lanes 5–6, i.e., at a 5-fold excess). Some topoisomerase activity becomes evident from the heterogeneity of the pTZ_{sc} species, leading to a contribution of up to 30% of lower topoisomers together with the nicked variant. Since the binding of SAR_{800} is completely unaffected by a substantial excess of pTZ_{sc}, these species appear to bind independently of each other.

Single-Stranded DNA Interferes with the Binding of SAR DNA but Not with the Association of Supercoiled Plasmids. In addition to binding supercoiled and linear SAR DNA, the scaffold contains sites for single-stranded DNA.

Figure 4A shows the relationship between the sites for supercoiled and single-stranded forms of pTZ. Since we had to trace the supercoils by hybridization toward a complementary probe (pCL), the outcome of a direct exposure (signals for SAR_{800} and PTZ_{lin} only in the control lanes) is omitted. After the blotting and hybridization steps, exclusive binding of both SAR_{800} and pTZ_{sc} to the scaffold (lanes P) is seen while the nonbinding control (pTZ_{lin}) resides in the S fraction,

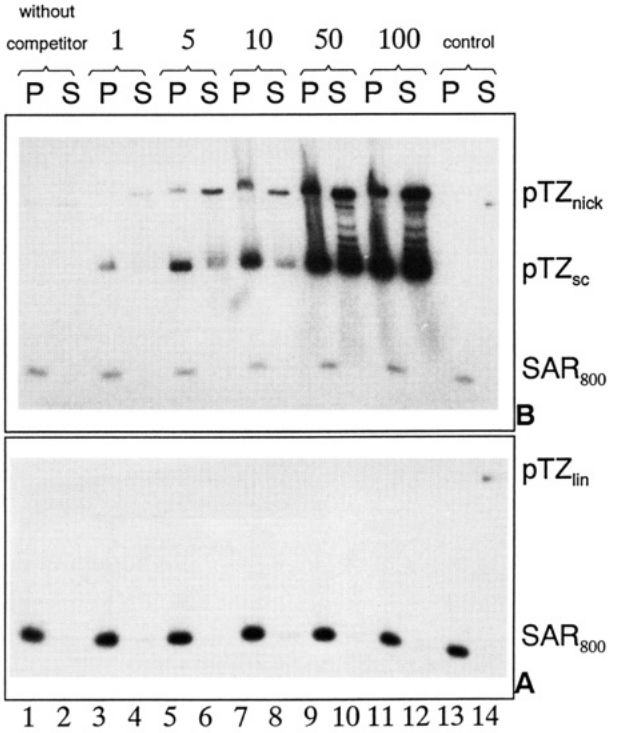

FIGURE 3: SAR_{800} retention in the P fraction even with excess supercoiled DNA (pTZ_{sc}). Scaffolds were isolated from two plates of mouse cells, digested with 300 units of *Bgl*II, and subsequently used for competition experiments. Radioactively end-labeled SAR_{800} was competed for by using an 1–100-fold excess of pTZ_{sc}. Autoradiographs of gels blotted onto a nylon screen are shown before (A) and after hybridization (B) with a nick-translated probe of pCL. A control assay (lanes 13 and 14) contained the binding SAR_{800} and the nonbinding pTZ_{lin} species as a reference. The sample volumes were determined from this control according to the modified equal counts approach and used accordingly for all other samples of P and S, respectively. SAR_{800}, *Eco*RI/*Hin*dIII fragment of pCL.

suggesting an affinity for pTZ_{sc} which equals or even exceeds that for SAR_{800} (lanes 1 and 2).

During the competition (lanes 3–14), binding of PTZ_{sc} remains unaffected by increasing amounts of heat-denatured, linear pTZ DNA (pTZ_{ss}) and even by the presence of a 15000-fold molar excess (based on nucleotides) of denatured *E. coli* genomic linear DNA. The association process involves an overnight incubation at 37 °C, causing the single-stranded competitor to form a range of partially renatured, slowly migrating species with considerable affinity for the scaffold (lanes 5 and 7). The appearance of bands comigrating with pTZ_{lin} in the supernate lanes 10 and 12 is ascribed to the saturation of sites for ssDNA, although some renaturation at these concentrations of single strands cannot be strictly ruled out.

In contrast to supercoils, the binding of linear, double-stranded SAR fragments is notably affected by an excess of the single-stranded competitor. The experiment has been performed at two levels of complexity: (i) addition of a 20–200-fold molar excess of single strands to SAR_{800}, leading to the gradual appearance of the S fraction and displacing about 50% of SAR DNA (autoradiograph not shown; data evaluated in Figure 5), and (ii) addition of a 20–2000-fold molar excess of single strands to a *mixture* of SAR_{800} and SAR_{2200}. SAR_{800} is a subfragment of SAR_{2200} [cf. fragments IV and I in Mielke et al. (1990)]. Owing to its extended length and the mode of SAR–scaffold interactions, SAR_{2200} has the higher affinity. This is immediately evident from the fact that part of SAR_{800} appears in the supernate due to internal competition (Figure

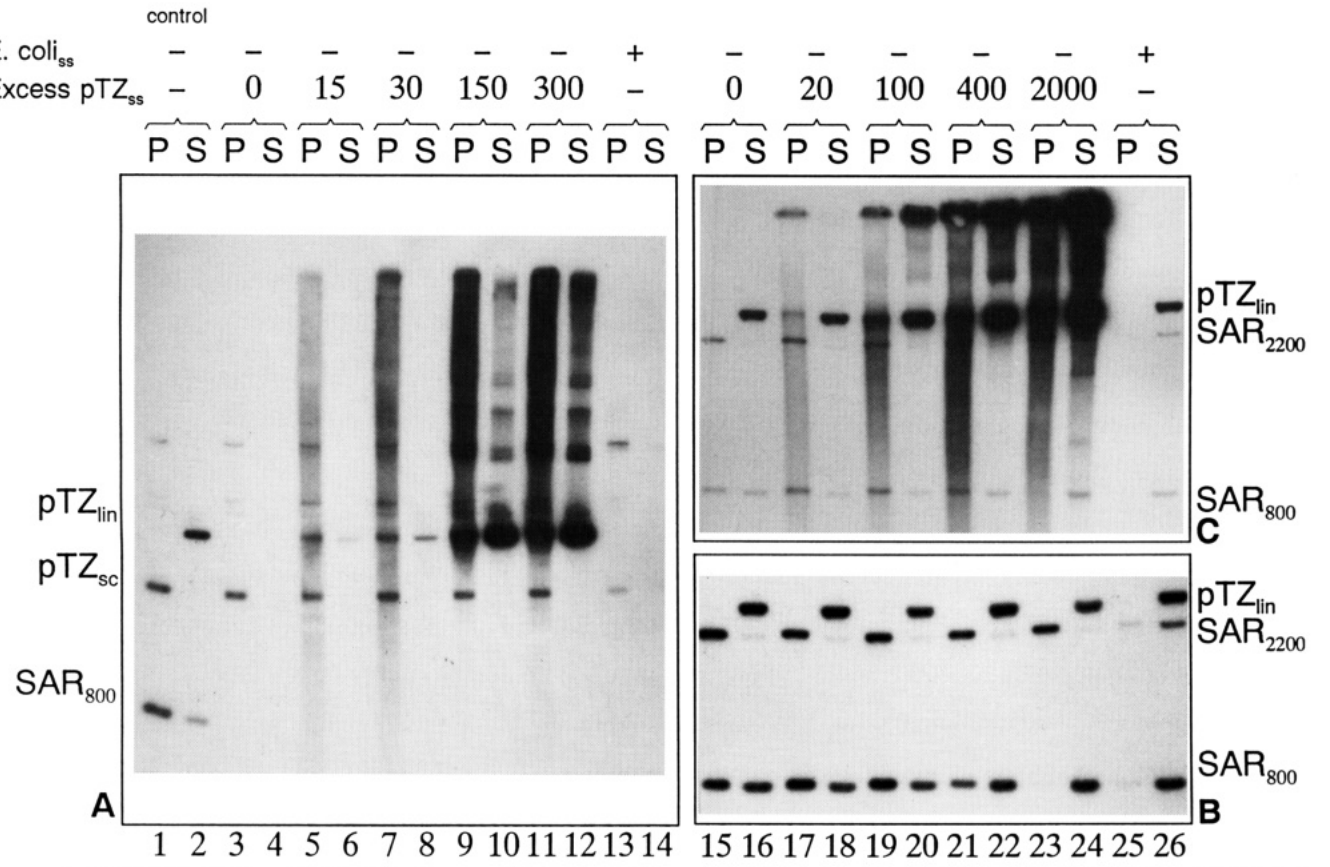

FIGURE 4: SAR DNA (SAR_{2200}/SAR_{800}) displacement from the nuclear scaffold by single-stranded DNA. Scaffolds were digested with the appropiate endonuclease(s) and used for the competition of (A) pTZ_{sc} or (B and C) SAR_{800}/SAR_{2200} with single-stranded, heat-denatured DNA (pTZ_{ss} or *E. coli* genomic DNA, respectively). The top numbers indicate the molar excess of competitor (double-strand equivalents). (A) Autoradiograph of a blot probed with nick-translated pCL. Nuclear scaffolds from two plates of mouse cells were digested with *Bgl*II. Lanes 1 and 2: Control of binding properties according to the modified equal-counts approach (see text). The remaining vials contained pTZ_{sc} without/with a 15–300-fold excess of pTZ_{ss}, as indicated on top. (B and C) Nuclear scaffolds, digested with *Bam*HI and *Sal*I, were supplied with SAR_{2200} and SAR_{800} and incubated without/with a 20–2000-fold excess of single-stranded pTZ_{ss}, as indicated above the autoradiographs. Autoradiographs of the same blot are shown before (B) and after (C) probing with nick-translated pCL. All incubations were carried out in the presence of genomic *E. coli* DNA (0.66 mg/mL) as an unspecific competitor, except for one vial in each experiment (lanes 13/14 and 25/26) that received an equivalent amount of heat-denatured *E. coli* DNA.

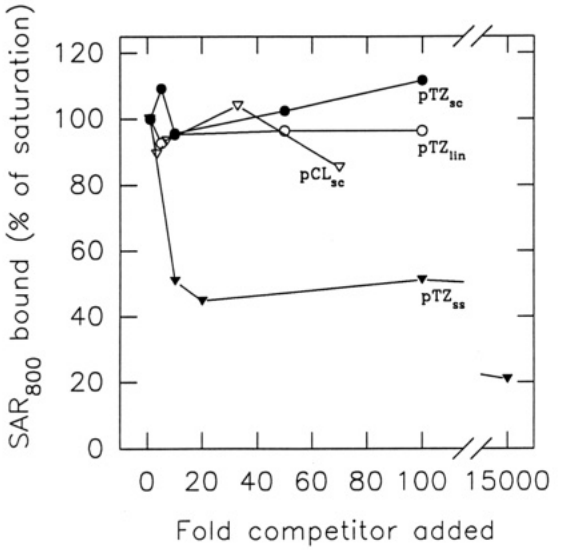

FIGURE 5: Summary of competition experiments. The amount of end-labeled SAR_{800} remaining in the P fraction after the application of various competitors is shown as the percent level attained in the absence of competitor (100%). The diagram summarizes the data from at least four independent experiments analogous to those in Figures 3 and 4.

4B, lanes 15 and 16). A 20–400-fold excess of single strands is sufficient to remove part of SAR_{800} from the scaffold while a 2000-fold excess is required for its complete displacement. SAR_{2200} remains bound even at this excess of competitor. Its displacement occurs only under the extreme conditions caused by denatured *E. coli* DNA (*E. coli*$_{ss}$) at a molar excess of 15 000 (Figure 4B, lanes 25 and 26).

Figure 5 compares some prominent displacement curves within a range of competitor concentrations that is covered by all experiments. It is quite evident from these data that, at a moderate excess of competitor, binding of SAR_{800} is affected by only single-stranded DNA, which releases about 50% of the total. Since a more complete displacement requires substantially higher amounts of competing DNA, this leaves the possibility that there are two classes of SAR-binding sites, one recognizing single-stranded regions and one specific for other structural features.

Proteins Recognizing Linear SAR Fragments and Supercoiled Plasmids Can Be Extracted Differentially. For SAR-binding experiments, binding sites first have to be uncovered by extracting soluble chromatin proteins. 3,5-Diiodosalicylate (LIS) is a mild detergent that is commonly used to achieve this under close to physiological salt concentrations. Systematic variation of the detergent's concentration showed that binding sites are first uncovered and then lost under more stringent conditions (Figure 6A).

The curve labeled Ia in Figure 6A reflects the standard conditions maintained for all previously described experiments. Under these conditions, the scaffold contains about 15000–

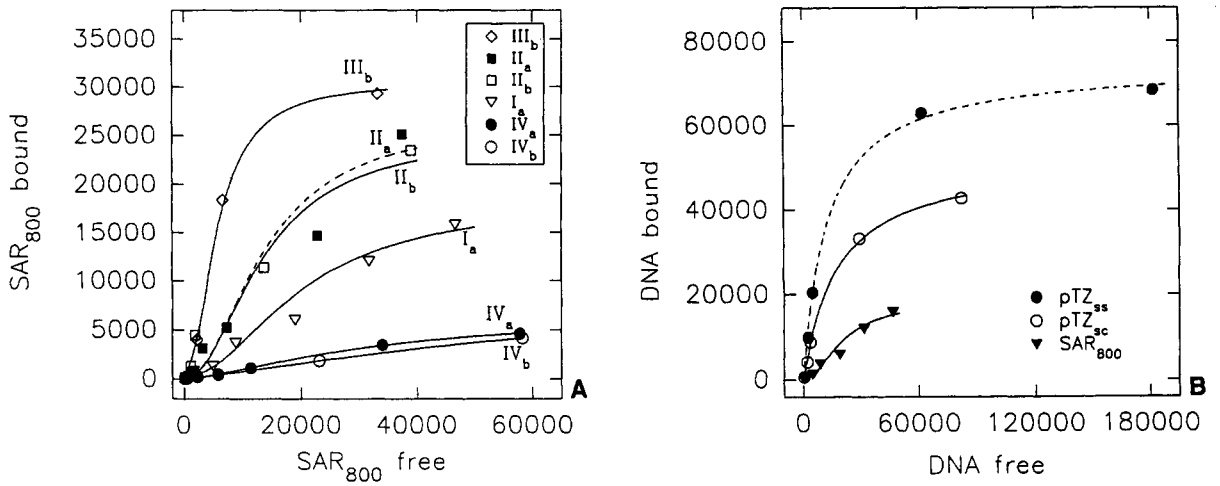

FIGURE 6: Number of binding sites in a scaffold preparation varying with degree of extraction and specific for the kind of DNA added. (A) Nuclear scaffolds were isolated from mouse cells using different LIS concentrations and extraction conditions (cf. Materials and Methods) and titrated with end-labeled SAR_{800}. After P/S separation, the radioactivity retained in the P fraction was expressed in copies bound per nuclear equivalent. Roman numerals (I–III) mark successively extracted preparations which still bind DNA; IV represents overextracted preparations which have lost their DNA-binding capability. Indexes mark experiments performed in the absence (a) or in the presence (b) of equimolar amounts of supercoiled plasmid DNA as a potential competitor. The protein contents (percent total nuclear protein) of the preparations were as follows: I_a (57.5%), II_a (44.9%), II_b (38.3%), III_b (29.9%), IV_a (6.4%), and IV_b (8.3%). The binding cooperativity, characterized by the ratio of K_{d1}/K_{d2}, was as follows: I_a (60.5), II_a (11.4), II_b (4.9), III_b (13.3), IV_a (2.8), and IV_b (1.0). (B) Titration curves of pTZ_{ss} (●) and pTZ_{sc} (○ taken from analyses like those in Figures 3 and 4); SAR_{800} is the same as curve I_a in A. The autoradiographs were scanned and the values obtained are expressed as DNA free vs DNA bound.

20000 accessible binding sites for the 800-bp SAR element and an analysis of many such experiments suggests that binding is a cooperative process [cf. detailed analyses in Mielke et al. (1990)]. Under the same conditions, there are about 40 000 binding sites for supercoiled DNA and 60 000 sites for single-stranded DNA, both of which consistently displayed hyperbolic binding characteristics (Figure 6B).

If scaffold preparations are extracted more extensively, more SAR sites are uncovered (Figure 6A, traces II and III) before the responsible protein(s) is (are) extracted and SAR binding is abolished (traces IV). As expected for two independent sets of binding sites, the binding of SAR DNA remains unaffected by the simultaneous presence of supercoiled DNA (compare traces IIa and IIb or IVa/IVb).

For Figure 7, extractions were performed close to and beyond the point where the SAR-binding property is lost. It is seen that at 12.5 and 25 mM LIS, i.e., concentrations where SAR binding is fully abolished, the binding of supercoiled topoisomers is still complete, again emphasizing the distinct nature of these sites.

DISCUSSION

The interaction of the nuclear matrix or scaffold with DNA has been ascribed to a number of properties [von Kries et al. (1990, 1991) and references therein]. Laemmli and coworkers have emphasized the importance of oligo(dA) tracts for the recognition of histone H1 and topoisomerase II, which are both major components of the scaffold (Izzauralde et al., 1989; Käs et al., 1989). At the other extreme, we have elaborated the relevance of processes causing the separation of strands (Bode et al., 1992). Such a property is not compatible with the presence of oligo(dA) tracts (Kohwi-Shigematsu et al., unpublished results), but is found in the case of certain motifs comprising runs of $(AT)_n$ if they are present in an appropriate, generally AT-rich, environment (Kohwi-Shigematsu & Kohwi, 1990; Bode et al., 1992). Other motifs may form an unwinding center, such as an extensive run of $(GA)_n$ in a functional SAR element isolated from plants (T. Kohwi-Shigematsu and J. Bode, unpublished results). $(GA)_n$ tracts are also part of certain DNAse I hypersensitive sites, as found in an enhancer or adjacent to a promoter (Petterson et al., 1990; Lu et al., 1992, 1993).

By using an unwinding center of the $(AT)_n$-type and a closely related mutant that had lost its unwinding capacity, Dickinson et al. (1992) screened for and cloned an 85.9-kDa "special AT-rich binding protein" (SATB1), the binding of which depends on this feature. Interestingly, this protein recognizes the full set of native SAR sequences from animals and plants that are presently available to us (about 20 species), but discriminates against poly(dA) and related non-unwinding polynucleotides. Other proteins that recognize a variety of S/MAR elements according to more or less undefined structural features are a 95-kDa protein (ARBP; von Kries et al., 1991), 120-kDa proteins [SAF-A, cf. Romig et al. (1992); SP120, cf. Tsutsui et al. (1993)], and a component of the nuclear lamina (lamin B_1; Ludérus et al., 1992).

In an effort to better understand the correlation between the mode of binding and the positive effects SAR sequences exert on the transcriptional properties of stably integrated genes, we have initiated experiments to identify the binding mode(s) and proteins responsible for the *in vivo* effects. As a first step toward this end, the present investigation explores the binding characteristics of the standard scaffold preparations which have been used sucessfully for screening functional SAR elements (Mielke et al., 1990). Any deviation from this protocol can lead to further uncovering or loss of SAR sites, as demonstrated in Figures 6A and 7.

Originally unexpected, we observed that SAR sequences are not recognized in the context of a negatively supercoiled plasmid; such a plasmid is bound irrespective of its sequence contents (Figure 1B). A general affinity of scaffolds for supercoils was first described by Tsutsui et al. (1988). Contrary to our results, they concluded that binding depended on the exposure of single-stranded portions, as supercoils were competitive with and could be replaced by single-stranded DNA. Since topoisomerase functions were obviously destroyed in their scaffold preparation while they are clearly maintained in the present ones (Figures 1A and 3B), we

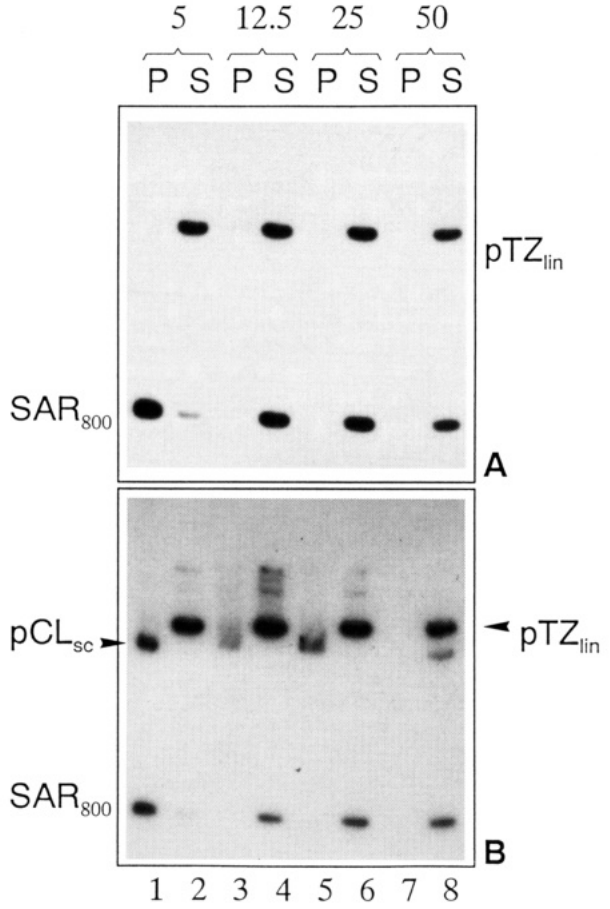

FIGURE 7: Binding specifity of nuclear matrix varying with degree of extraction. Autoradiographs of a gel blotted onto a nylon screen before (A) and after (B) hybridization with nick-translated pCL. Nuclei from mouse cells were extracted sequentially with increasing LIS concentrations (5–50 mmol/L) as indicated. Each scaffold fraction was supplied with 20 fmol of supercoiled plasmid (pCL_{sc}) and 10 fmol of an end-labeled *Bam*HI/*Hin*dIII digest of the same plasmid. After the probes were separated into P and S fractions, they were submitted to the usual DNA purification procedure. The application of equivalent fractions of P and S to the gel followed the modified equal counts protocol.

tentatively ascribe the strong recognition of supercoils (Figures 4A and 7B) to topoisomerase II, which is a major component of the complete scaffold. Kinetic studies have demonstrated that nicked DNA is an obligatory intermediate in the enzyme's pathway for religating double-strand breaks (Osheroff et al., 1991). Therefore, the contribution of both nicked and partially relaxed, supercoiled forms in the final scaffold–DNA mixture can be ascribed to the enzyme's action. In our hands, nicking of supercoils occurs at random, i.e., in a sequence-independent fashion (V. Kay, unpublished results), which would fit well with the preferential recognition by topoisomerase II of plectonemic DNA strands rather than consensus sequences (Osheroff et al., 1991).

In our system, the binding of linear SAR elements occurs completely independently of the association of supercoils, as shown by the titration of two nonoverlapping sets of sites (Figure 6), the different competition by single strands (Figure 4), and the differential extractability of the binding proteins (Figure 7). A contribution of single strands to the binding of linear SAR DNA has been mentioned before (Gasser et al., 1989). Our data provide direct evidence that this is partly true, i.e., for about one-half of the binding sites, as these are easily competed for by single-stranded DNA, whereas the remainder requires substantially more if it is affected at all (cf. Figure 5). This leaves the question of the nature of a second binding contribution, which is exemplified by the proteins ARBT, SAF-A/SP120, and SATB1 described above, which do not recognize single strands, require multisite attachment to DNA with a minimum length of 100–300 bp, and can conceivably give rise to the observed binding cooperativity. In our hands, cooperative binding is a property only of linear SAR elements, while the saturation functions of supercoiled and single-stranded DNA are simply hyperbolic.

We are presently trying to separate these binding modes by the dissection of long SARs and by oligomerization of their components, according to the approach introduced by Mielke et al. (1990) and Bode et al. (1992). These artificial elements will then be tested in parallel for their interaction with scaffold proteins and their enhancing effects in transcriptional assays. This approach will also address the question of whether these protein–DNA contacts are responsible for conferring the position-independent expression of transgenes, which has been established in some systems (Stief et al., 1989; Phi-Van et al., 1990; Breyne et al., 1992) but not in others (Kellum & Schedl, 1992; Allen et al., 1993).

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