Specialized Chromatin Structure Domain Boundary Elements Flanking a *Drosophila* Heat Shock Gene Locus Are Under Torsional Strain *in Vivo*†

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ABSTRACT: An *in vivo* assay employing psoralen cross-linking was used to investigate the presence of unrestrained supercoiling in DNA sequences located in nontranscribed regions flanking the 3′ ends of the pair of divergent heat shock protein 70 (hsp70) genes at locus 87A7 of *Drosophila*. Two of the regions examined contain sequences comprising the previously defined specialized chromatin structure elements (*scs* and *scs*′). Both of these putative chromosomal domain boundaries exhibited very similar levels of unrestrained negative supercoiling that remained high regardless of the transcriptional status of the hsp70 genes. The steric accessibility of the *scs* region before heat shock was 3-fold higher than either flanking region (consistent with its previously documented DNase I hypersensitivity); this increased an additional 2-fold following hsp70 gene activation without a concomitant rise in the accessibility of flanking regions. Most notably, a sequence which lies outside the presumed 87A7 domain, as defined by the centromere-proximal *scs* element, exhibited no detectable torsional tension regardless of gene activity in the domain. A sequence located just inside the *scs* region displayed a low level of tension that was also essentially unaffected by transcription, consistent with data obtained previously for a similarly situated fragment at the centromere-distal *scs*′ location. The existence of a highly localized region of supercoiling within the *scs* and *scs*′ sequences might be related to their activity *in vivo* as insulators of chromosomal position effects in *Drosophila*.

The chromosomes of eukaryotic organisms are believed to be organized into independent loops comprising topologically closed domains. Current models of eukaryotic chromatin organization, based on a wide variety of biochemical and genetic evidence, suggest that looped chromatin domains are anchored at intranuclear attachment points, perhaps located on the nuclear matrix, and these attachments functionally insulate the loop from the effects of neighboring domains (Freeman & Garrard, 1992). This division of a chromosome into independent domains provides a framework on which to propose the possibility of topological regulation of different genes throughout the chromosome (Esposito et al., 1988). One of the most well-characterized domains in eukaryotes is locus 87A7 of *Drosophila melanogaster*. This domain consists of approximately 12 kilobases (kb)[1] of DNA containing two divergent hsp70 transcription units (Goldschmidt-Clermont, 1980), flanked by specialized chromatin structures (*scs* and *scs*′) that were originally identified as constitutive DNase I hypersensitive sites (Udvardy et al., 1985). In a series of elegant P-element transformation experiments, these *scs*/*scs*′ regions were found to act as effective insulators both of general chromosomal position effects and of the interaction between an enhancer and a promoter when interposed between them (Kellum & Schedl, 1991, 1992). Insulator sequences have also been identified upstream of the chicken β-globin gene locus (Chung et al., 1993). Other than their unique chromatin structure, which is frequently diagnostic of a nonnucleosomal DNA−protein interaction, rather little is definitely known about insulator sequences or their mechanism of action *in vivo*. However, a feature of particular interest is that the *scs* sequences do not apparently display *in vitro* binding to a nuclear matrix or scaffold (Mirkovitch et al., 1984), an assay in which a positive signal is typically used to characterize and define a class of A/T-rich elements known as matrix/scaffold attachment regions (MARs/SARs). Since some sequences that display MAR/SAR activity *in vitro* are capable of conferring position-independent expression to associated transgenes, it seems likely that *scs* sequences represent a distinct class of insulator, and potentially domain boundary, elements [see Felsenfeld (1992) for a review].

Recent studies using psoralen photobinding as an assay for unrestrained supercoiling have demonstrated that, in spite of bulk chromatin being torsionally relaxed, transcriptionally active genes can be organized with unrestrained negative supercoiling. Coding regions of the human 18S rRNA and DHFR genes and of the *Drosophila* 18S rRNA and hsp70 genes exhibit unrestrained negative supercoiling in living cells (Ljungman & Hanawalt, 1992; Jupe et al., 1993). The

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[1] Abbreviations: bp, base pairs; kb, kilobase; XL, cross-link; NXL, non-cross-linked; HS, heat shock; NHS, non-heat shock; hsp70, heat shock protein 70; psoralen, 4′,5′,8-trimethylpsoralen; MAR, matrix attachment region; SAR, scaffold attachment region; *scs*, *scs*′, specialized chromatin structure.

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hsp70 genes displayed a constant level of torsional tension in their DNA whether or not the genes were transcribed. Quantitative analysis of psoralen photo-cross-linking also demonstrated an increase in the steric accessibility of the coding region to this chemical probe upon hsp70 gene activation, consistent with the results of earlier DNase I analyses (Wu et al., 1979). Previously we showed that, in contrast to the constitutively high level of tension over the hsp70 transcription units, a region located immediately inside the 87A7 domain and just flanking the *scs'* element contained a low level of unrestrained supercoiling whether or not the hsp70 genes were active (Jupe et al., 1993). In the present study, we have extended the analysis of unrestrained negative supercoiling throughout the microdomain containing the 87A7 locus. We show that both the *scs* and *scs'* elements are wound with a level of torsional tension similar to that observed in the hsp70 coding region. Moreover, as demonstrated previously, other noncoding DNA sequences between the *scs* and *scs'* sequences contain a low level of unrestrained torsional tension. However, in a region outside the transcriptionally competent 87A7 domain, i.e., beyond the *scs* element, we find no evidence for the presence of torsional tension, a result characteristic of the bulk of *Drosophila* chromatin (Sinden et al., 1980).

EXPERIMENTAL PROCEDURES

Cell Culture. Schneider line 3 *Drosophila* cells were maintained in serum-free Excell-400 medium (JRH Biosciences) in T-75 flasks. Cultures were scaled up for the psoralen cross-linking assays by transferring them to spinner flasks containing Excell-400 medium supplemented with 50 μM bromodeoxyuridine (BrdUrd) (Jupe et al., 1993).

Cell Treatments. Cells grown at 25 °C to a density of (3−5) × 10^6 cells/mL were harvested and used either immediately (non-heat-shocked, NHS) or after incubation at 37 °C for 30 min to induce transcription of the hsp70 genes (heat-shocked, HS). The cells were rapidly chilled, and all subsequent treatments were carried out on ice. Aliquots of either intact cells or cells containing nicked domains (see below) were then subjected to psoralen cross-linking. Domains of supercoiling were nicked *in vivo* by exposing the cells grown in BrdUrd to 313 nm light for 20 min using a high-pressure mercury lamp filtered through a K_2CrO_4/NaOH solution (Sinden & Pettijohn, 1982; Jupe et al., 1993). The nicking dose required to maximally reduce the rate of cross-linking was determined as described previously (Jupe et al., 1993). For cross-linking, psoralen (4,5',8-trimethylpsoralen) was added to a final concentration of 0.2 μg/mL, and cells were exposed to 6 kJ m^{-2} of 360 nm light (within the linear dose range of cross-link formation). The DNA was then purified using standard procedures as described previously (Jupe et al., 1993). Although there could be a small increase in negative supercoiling from temperature-induced changes in the helix twist on non-nucleosome-associated DNA, earlier analyses did not detect measurable supercoiling in bulk chromatin when assayed by psoralen photobinding at 4 °C (Sinden et al., 1980). Moreover, our previous analysis of the hsp70 coding regions showed no measurable differences in supercoiling when cells were treated with psoralen at 4, 25, or 37 °C (Jupe et al., 1993).

Analysis of Psoralen Cross-Linked DNA. DNA purified from the cross-linked samples was restricted, glyoxal-denatured, separated by agarose gel electrophoresis, and analyzed by Southern blot hybridization as previously described (Jupe et al., 1993). The various fragments used as hybridization probes were from the hsp70 centromere-proximal (*scs* and flanking region) and centromere-distal downstream regions (*scs'*), and were purified from plasmid subclones obtained from A. Udvardy and P. Schedl (Udvardy et al., 1985). The individual fragments were obtained by restriction enzyme digestion, separation on 1% agarose gels, excision of the desired bands, and purification using the Gene Clean Kit (Bio 101). Radioactive labeling was performed by random hexamer priming (Feinberg & Vogelstein, 1983).

The washed filters were exposed to a phosphor screen for 4−20 h, and band intensities were quantitated with a Molecular Dynamics PhosphorImager using ImageQuant Software. Autoradiograms were also made for illustrative purposes using preflashed Kodak XAR-5 film. The cross-links per kilobase of DNA for each fragment were calculated as previously described (Jupe et al., 1993).

RESULTS

We have previously developed a psoralen cross-linking assay to quantitate relative levels of unrestrained supercoiling *in vivo* across the hsp70 genes and some flanking regions. This assay is based on comparing rates of psoralen cross-linking in intact cells with those in cells where potential tension in chromosomal domains has been relaxed by nicking the DNA *in vivo* (Jupe et al., 1993). Since the rate of psoralen photobinding is proportional to the level of unrestrained superhelical tension (Sinden et al., 1980), a ratio of cross-linking in intact domains relative to that in nicked domains provides an estimate of the level of torsional tension in chromosomal DNA. This strategy also controls for any changes in steric accessibility of the chromatin resulting from a redistribution of DNA−protein associations that might be caused by the processes of transcription or replication. Because of the reliance on comparative ratios, the assay is also insensitive to differences in the inherent rate of cross-linking to a restriction fragment, which would be a function of base composition and the frequency of 5'TA dinucleotides (Esposito et al., 1988). For a detailed discussion of the rationale of the assay, see Sinden et al. (1980, 1982), Sinden and Ussery (1992), and Jupe et al. (1993). Here we expand the *in vivo* analysis of the hsp70 domain by focusing on the state of unrestrained supercoiling at and around regions of the microdomain that serve as the putative functional boundaries.

The various restriction fragments flanking the divergent hsp70 genes at locus 87A7 that were investigated in this study are shown in the map in Figure 1. The *Eco*RI/*Bgl*II fragment is 5' to the proximal *scs* element, which places it outside the putative functional domain boundary delineated by the *scs* sequence (Udvardy et al., 1985; Kellum & Schedl, 1991, 1992). The *Bgl*II/*Bam*HI fragment contains the entire *scs* element together with some flanking sequence (Udvardy & Schedl, 1993). The *Bam*HI/*Eco*RI fragment lies immediately adjacent to the *scs* fragment but just inside the putative domain boundary. We have also assayed the *scs'* element which is included within the *Eco*RI/*Bam*HI fragment at the distal end of the locus (Udvardy et al., 1985).

The results of a psoralen cross-linking analysis on fragments derived from the proximal end of the locus performed

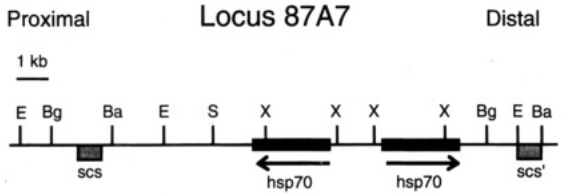

FIGURE 1: Map of hsp70 locus 87A7 showing the regions examined in this study. The *scs* and flanking proximal downstream regions (indicating location relative to the centromere) and the *scs′* region studied are defined by the restriction sites shown (Udvardy et al., 1985). The remainder of the locus consists of a pair of divergently transcribed genes (solid boxes with arrows showing the direction of transcription) and flanking regions which were examined in a prior study (Jupe et al., 1993). The *scs* and *scs′* elements flanking the locus are depicted by shaded boxes. E, *Eco*RI; Bg, *Bgl*II; Ba, *Bam*HI; S, *Sal*I; X, *Xba*I.

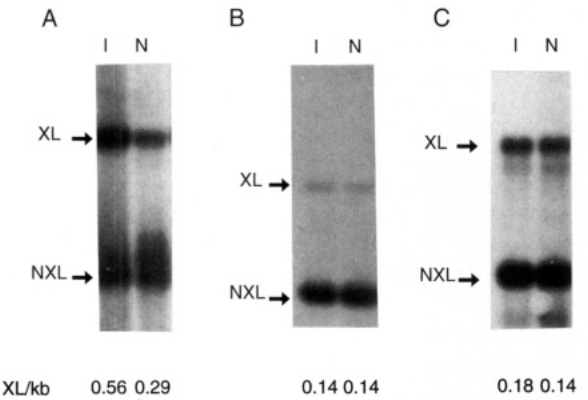

FIGURE 2: Psoralen cross-linking *in vivo* in the proximal *scs* and flanking regions. Cells with chromosomal domains either intact (I) or nicked (N) were cross-linked with psoralen as described under Experimental Procedures to characterize torsional tension. The non-cross-linked (NXL) and cross-linked (XL) bands separated by gel electrophoresis are indicated on the left-hand side of each panel. The cross-links per kilobase (XL/kb) are shown below each lane. (A) Samples were double-digested with *Bgl*II/*Bam*HI, denatured, and analyzed as described under Experimental Procedures. The corresponding *scs* fragment (Figure 1), gel-purified from restricted plasmid DNA, was used as the probe in this experiment. (B) Samples were digested with *Eco*RI/*Bgl*II and probed with the corresponding gel-purified fragment prepared from a plasmid. (C) Samples were digested with *Bam*HI/*Eco*RI and probed with the corresponding gel-purified fragment. The psoralen cross-linking analyses were all from heat-shocked samples.

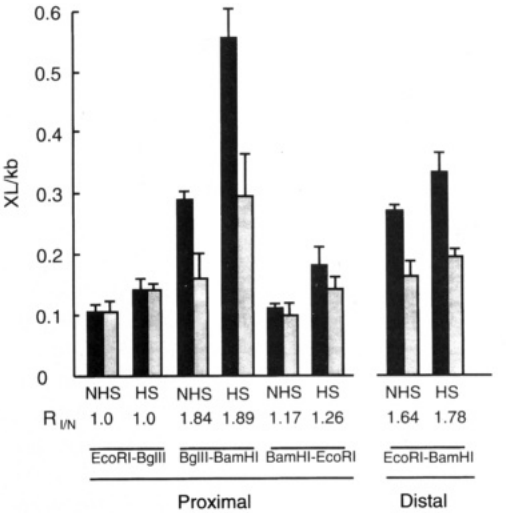

FIGURE 3: Summary of the psoralen cross-linking rates of restriction fragments flanking the hsp70 genes at locus 87A7 in intact and nicked samples from non-heat-shocked and heat-shocked cells. The level of cross-linking per kilobase is plotted on the vertical axis. The solid bars show the level of psoralen cross-linking *in vivo* to restriction fragments derived from intact cells, and the shaded bars depict the level of cross-linking to DNA in cells that were subjected to the nicking treatment. Each of the bars represents the average of three cross-linking levels determined from two (NHS) or three (HS) independent cell preparations. The standard deviation for each is shown by the error bars. The ratio of the mean cross-linking levels calculated for intact/nicked domains ($R_{I/N}$) is presented for each of the four restriction fragments surveyed. The locations of these fragments at locus 87A7 are shown in Figure 1.

on heat-shocked cells are displayed in Figure 2. Panel A shows the data obtained for the *Bgl*II/*Bam*HI fragment (Figure 1) which includes the proximal *scs* element. In intact cells (lane I), 0.56 cross-link per kilobase was introduced under the standard conditions of the assay. Following nicking (lane N), it is evident that the signal in the cross-linked fraction (XL) has decreased and that the signal in the non-cross-linked fraction (NXL) has increased compared to the respective signals in intact cells (compare lane I with lane N). Quantitatively, there was an approximate 2-fold reduction in cross-links per kilobase to 0.29 following nicking. Such a result strongly suggests that torsional tension is present *in vivo* across the fragment containing the *scs* element. Results for the *Eco*RI/*Bgl*II fragment, which lies outside of the functional domain defined by the *scs* element, are displayed in Figure 2B. In contrast to the results for the fragment shown in panel A, a quantitative comparison of lane I to N shows identical values of cross-links per kilobase. This is consistent with the *Eco*RI/*Bgl*II fragment in the chromosome being devoid of any torsional tension. The *Bam*HI/*Eco*RI fragment located inside the *scs* sequence showed a small reduction in cross-linking upon nicking (Figure 2C). Thus, torsional tension is present across the *scs* region, but the regions flanking it are either completely relaxed (*Eco*RI/*Bgl*II) or contain only a low level of unrestrained supercoiling (*Bam*HI/*Eco*RI). Moreover, the *scs* fragment exhibits significantly higher overall steric accessibility to psoralen than either flanking fragment, consistent with the expectation from DNase I digestion studies of these sequences in chromatin (Udvardy et al., 1985; Udvardy & Schedl, 1993) as discussed below.

The results of several experiments similar to those shown in Figure 2, performed both on these regions as well as on the *scs′* element at the distal end of the locus, are summarized in Figure 3. In each case, both non heat shock and heat shock conditions were examined to determine the effect of transcription of the hsp70 genes on torsional tension in these nontranscribed regions. The data obtained from these cross-linking experiments are indicative of the complex relationship between differential accessibility and torsional tension in chromosomal DNA *in vivo* [see Sinden et al. (1982) and Jupe et al. (1993) for discussion]. In Figure 3, the cross-linking level per kilobase for intact domains (solid bar) is compared to the level for the same domain measured subsequent to relaxation by nicking for 20 min (shaded bar). The height of the solid bars represents measurements which include effects derived both from the presence of torsional tension and from general DNA accessibility toward psoralen in intact cells. The height of the shaded bars provides a measure of only the DNA helix accessibility to psoralen under relaxed conditions since any potential unrestrained DNA supercoiling will have been released by the introduction of nicks into the

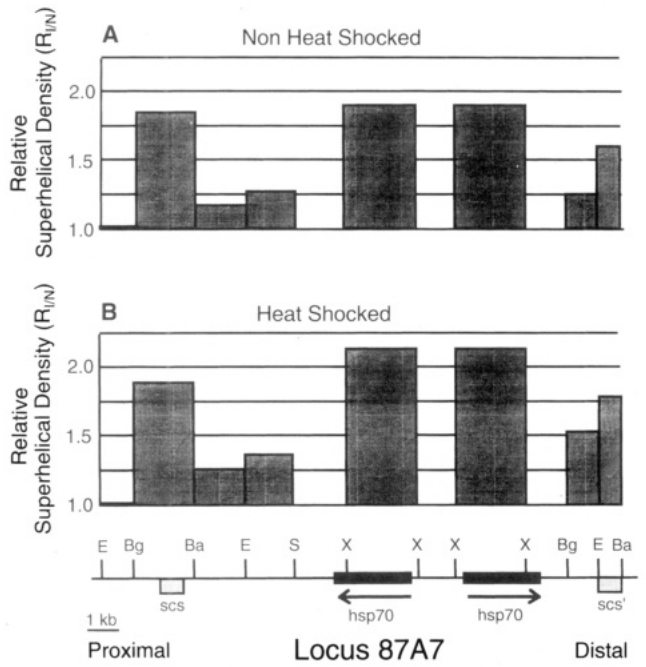

FIGURE 4: Overview of the torsional tension levels detected across locus 87A7. $R_{I/N}$ values for the four fragments examined in this study under non heat shock conditions (A) and heat shock conditions (B) are presented along with those determined for four fragments in our previous study (Jupe et al., 1993). The width of the bar corresponds to the length of the fragments analyzed. The map below shows the location of each of the fragments as well as the structural and functional landmarks of the 87A7 locus.

DNA. Thus, the ratio of the cross-linking rate in intact (solid bar) versus relaxed (shaded bars) chromosomal domains ($R_{I/N}$ shown at the bottom of Figure 3) provides a measure of unrestrained DNA supercoiling averaged over the DNA restriction fragment. Comparing the relative ratio between fragments reveals the differential partitioning of unrestrained supercoiling across the locus (Figures 3 and 4). The higher the value of $R_{I/N}$, the greater the level of unrestrained supercoiling in a fragment. The levels of tension present in all nontranscribed regions examined are essentially unaffected by transcription of the hsp70 genes in the domain (compare Figures 4A and 4B). A low level of tension is present in the *Bam*HI/*Eco*RI fragment that lies adjacent to the *scs* element but within the domain. This low level is comparable to that which we determined for the immediately adjacent *Eco*RI/*Sal*I fragment analyzed previously (Jupe et al., 1993; see Figure 4). Of particular interest is the complete lack of tension in the *Eco*RI/*Bgl*II fragment which is located outside of the domain. This is the only fragment we have assayed that is devoid of tension. In addition, the accessibility of the fragment remains the same regardless of the conditions of gene activity within the domain. Thus, this region is in a relaxed and inaccessible condition in the genome, suggesting that it is wrapped tightly in nucleosomes and reflects the condition characteristic of bulk chromatin (Sinden et al., 1980).

The level of torsional tension in restriction fragments encompassing the *scs* and *scs′* elements was almost as high as that observed in the gene coding regions. Although the *scs* and *scs′* fragments are not transcribed, their overall accessibility to psoralen, but not level of supercoiling, increased significantly following heat shock induced transcription of the locus. These changes are likely to be mechanistically related to a unique genetic function exhibited by such elements within the *Drosophila* chromosome.

DISCUSSION

We have previously discussed in detail the observation of constitutive unrestrained supercoiling (torsional tension) across the *Drosophila* hsp70 coding region (Jupe et al., 1993) and its potential relationship to the data of Lis and colleagues concerning the presence of elongationally paused transcription complexes on these genes even in the transcriptionally inactive condition (Rougvie & Lis, 1988, 1990). The current study has significantly extended our observations on the presence and persistence of unrestrained supercoiling *in vivo* across chromosomal locus 87A7 of *Drosophila*, a region encompassing a divergently transcribed pair of these hsp70 genes. Figures 4A and 4B summarize the levels of unrestrained supercoiling present throughout the locus under both transcriptionally inactive and active conditions, respectively [this work and Jupe et al. (1993)]. Most importantly, these data reveal that tension exists at heterogeneous levels through the domain. Significantly, a high level of superhelical tension was detected in restriction fragments encompassing the *scs* and *scs′* elements, sequences defined genetically as insulators of enhancer-mediated position effects (Kellum & Schedl, 1991, 1992) and proposed to function as boundary elements for the 87A7 domain (Udvardy et al., 1985). Restriction fragments flanking the *scs* and *scs′* elements displayed significantly lower levels of tension. The observation of localized tension at the putative boundaries to the hsp70 domain adds an intriguing new twist to the litany of interesting features associated with this locus.

Previous studies have shown that bulk chromatin is torsionally relaxed (Sinden et al., 1980). The small number of eukaryotic gene coding regions so far analyzed *in vivo* by psoralen photo-cross-linking have, by contrast, exhibited varying degrees of unrestrained torsional tension (Ljungman & Hanawalt, 1992; Jupe et al., 1993; E. R. Jupe, R. R. Sinden, and I. L. Cartwright, unpublished data). However, the *Eco*RI/*Bgl*II fragment immediately adjacent to *scs* (but lying outside of the presumed 87A7 domain boundary) displayed no detectable tension whatsoever. Interestingly, no genes (either active or inactive) have been identified in this torsionally relaxed *scs*-proximal region (Udvardy & Schedl, 1993). It seems reasonable to propose that such genetically "inactive" regions or domains of chromatin will be constitutively relaxed. Given that greater than 90% of higher eukaryotic chromatin is likely to be genetically "inactive", it would therefore not be surprising that eukaryotic chromosomes, on average, display no net level of negative supercoiling (Sinden et al., 1980). We are currently extending the psoralen assay to probe other known "inactive" chromatin domains to determine the generality of this observation.

In contrast to regions immediately flanking the *scs* element, a *Bgl*II/*Eco*RI fragment lying immediately adjacent to the *scs′* region, but within the 87A7 domain, displayed substantially higher (i.e., quantitatively intermediate) levels of tension. A preliminary examination of the region flanking the other side of *scs′* also detected constitutive tension (unpublished observation). This 87A7-distal flanking region contains a constitutively expressed gene located close to, and transcribed toward, *scs′* (Udvardy et al., 1985; Udvardy &

Schedl, 1993). Therefore, the tension observed in the *scs′* element, or that outside the hsp70 microdomain, could be partly the result of this transcriptional activity. However, even in this region of complex chromatin organization and transcription, the *scs′* element may be functioning as an effective insulator of superhelical tension transmission, given the reduction in tension in the *BglII/Eco*RI fragment inside the distal portion of the domain (Figure 4). This potential ability of the *scs/scs′* elements to insulate the propagation of unrestrained negative supercoiling is dramatically indicated at the proximal end of the locus where a very abrupt boundary between high levels and zero tension is present.

The *scs/scs′* elements have not been reported to exhibit the *in vitro* matrix or scaffold binding properties characteristic of A/T-rich sequences known as MARs or SARs, and thus they may represent a distinct (although possibly related) functional class of chromosomal DNA elements. Several MAR/SAR elements have been found at locations that could formally be considered as chromosomal domain boundaries by several criteria, including their coincidence with regions of highly decreased sensitivity to DNase I (Strätling et al., 1986; Levy-Wilson & Fortier, 1989) and their ability to confer copy number-dependent, position-independent expression to linked transgenes in both permanently transformed cells and transgenic animals (Stief et al., 1989; Bonifer et al., 1990; Phi-Van et al., 1990). On the other hand, unlike the *scs/scs′* elements or a functionally similar element characterized at the chicken β-globin locus (Chung et al., 1993), at least some MAR/SAR elements are not genetically neutral since, while inactive in transient assays, they have displayed distinct enhancing activity on linked genes when assayed in permanently transformed cells (Stief et al., 1989; Klehr et al., 1992; Bode et al., 1992). Studies on the ease of unwinding under superhelical stress of some of these same MAR/SAR elements (Kohwi-Shigematsu & Kohwi, 1990, Bode et al., 1992) have led to the suggestion that one of their functions could be as a reservoir for torsional stress generated (e.g., by transcription) within a given domain (Bode et al., 1992). While we have uncovered a localized region of superhelical tension at the *scs* and *scs′* elements, it is not likely to have been generated via transcriptional processes at 87A7 since it is present under both non heat shock and heat shock conditions. Moreover, in the case of the *scs* element, there is no other gene (at least currently known) outside the domain that could be involved in generating tension from the proximal direction. It will obviously be of interest to determine whether the presence of a localized domain of unrestrained tension at the *scs/scs′*-type sequences is linked mechanistically to their putative function as domain boundary and/or genetic insulator elements.

An important question concerns how tension at the 87A7 domain and, in particular, at the *scs/scs′* sequences might be established. An intriguing possibility has been raised by observations that hyperacetylation of core histones *in vitro* leads to release of a portion of the previously nucleosomally-restrained supercoils (Norton et al., 1989, 1990). Hyperacetylation has long been correlated with "active" chromatin: indeed, a recent study has shown that erythroid-specific histone hyperacetylation at the chicken β-globin locus comaps with the domain of heightened generalized DNase I sensitivity (Hebbes et al., 1994). Furthermore, the hyperacetylated region encompasses and extends just past a sequence at the 5′ end of the locus shown to act as a genetic insulator both in chick cells and in transgenic *Drosophila* (Chung et al., 1993). Therefore, although not yet determined, the 87A7 locus might lie in a hyperacetylated domain encompassing both the *scs* elements and the genes themselves. The constitutively "active" or "preset" nature of this chromatin domain [see Wallrath et al. (1994) for a review], together with our demonstration of the presence of unrestrained supercoils, is certainly strongly consistent with such a possibility. The nonuniform dispersion of the supercoils across the domain could represent regions of greater or lesser acetylation, preferential accumulation in sequences best able to accommodate them (such as in DNA unwinding elements), or some combination of the two. While the *scs/scs′* and MAR/SAR elements appear to show some contrasting features, it is interesting that Schlake et al. (1994) have reported a synergistic transcriptional enhancement when testing a MAR derived from the human β-interferon gene under conditions of core histone acetylation.

Inspection of Figure 4 revealed that regions over which the highest levels of tension are detected and constitutively maintained coincide remarkably well with sites where constitutive or inducible topoisomerase I and II activities have been reported for this locus (Rowe et al., 1986; Kroeger & Rowe, 1989, 1992; Udvardy & Schedl, 1991, 1993). This suggests that accessibility of chromatin to topoisomerase may be highly regulated in terms of both location and degree of enzymatic activity. While this proposition stems directly from the observed data, it is entirely consistent with increasing evidence that topoisomerase relaxing activity does not just passively gain access to any torsionally strained DNA structure *in vitro* or *in vivo* [for reviews, see Wang and Lynch (1993) and Dröge (1994)]. There is, of course, the possibility that active supercoiling of the locus is related to DNA gyrase-like topoisomerase activity; however, there remains no unambiguous demonstration of such an activity in eukaryotic cells, nor is it necessary to postulate one in order to account for our results.

Additional mechanisms for generating and/or stabilizing negative superhelicity in eukaryotic cells can be hypothesized, none of which can be either ruled in or ruled out without additional information. However, it is important to emphasize that realistic pathways for generating torsional tension do exist (Freeman & Garrard, 1992; Dröge, 1994), and it is no longer a serious objection that DNA gyrase activity has not been demonstrated in eukaryotic cells. Recent work on the ubiquitous class of eukaryotic HMG1/HMG2 proteins has led to suggestions that these chromosomally-associated molecules may play an important role in interacting with DNA that can assume distinctive structural or architectural features (such as four-way junctions, cross-overs, and cruciforms), as well as participate in bending formally linear duplex into negatively supercoiled conformations (Lilley, 1992). Ner and Travers (1994) have suggested that these proteins may play a chaperone-like role in mediating the conformational heterogeneity that may be required in numerous functional nucleoprotein complexes, in effect harnessing the inherent energy in DNA that is partitioned between writhe and twist. The participation of such proteins in the conformational heterogeneity of the 87A7 domain is of obvious interest.

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