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Stably maintained microdomain of localized unrestrained supercoiling at a *Drosophila* heat shock gene locus

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A psoralen crosslinking assay was utilized to detect localized, unrestrained DNA supercoiling (torsional tension) *in vivo* in *Drosophila* chromosomal regions subject to differential transcriptional activity. By comparing rates of crosslinking in intact cells with those in cells where potential tension in chromosomal domains was relaxed by DNA strand nicking, the contribution to psoralen accessibility caused by altered DNA–protein interactions (e.g. nucleosomal perturbations) was distinguished from that due to the presence of unrestrained supercoiling in a region of interest. The heat shock protein 70 (*hsp70*) genes were wound with a significant level of superhelical tension that remained virtually unaltered whether or not the genes were transcriptionally activated by thermal elevation. Constitutively expressed 18S ribosomal RNA genes also exhibited unrestrained superhelical tension at a level comparable with that across *hsp70*. In contrast, flanking regions downstream of each of the divergent *hsp70* genes at locus 87A7 exhibited substantially less tension. Thus the results point to the existence of stable, torsionally stressed topological domains within eukaryotic chromosomal DNA, suggesting that the relaxing action of topoisomerases is not ubiquitous throughout the nucleus but, in fact, is likely to be tightly regulated.

Key words: chromatin domains/DNA supercoiling/*Drosophila hsp70*/psoralen/gene expression

Introduction

Packaging linear DNA into chromosomes requires compaction of ~1000-fold in *Escherichia coli* and up to 10000-fold in human cells at metaphase (Dupraw, 1970). Such chromosomal organization leads to the partitioning of DNA into adjacent, independent loops of topologically closed domains (Benyajati and Worcel, 1976; Cook and Brazell, 1976; Igo-Kemenes and Zachau, 1977). Within these loops, supercoiling of the DNA may be differentially restrained by winding associations with chromosomal proteins. Release of supercoils from restraint can lead to the equilibration of supercoiling energy throughout the DNA helix as torsional tension, a condition of altered helical twist relative to that of relaxed DNA (Esposito and Sinden, 1989). Levels of torsional tension in a topological domain (or a portion thereof) may also be influenced by topoisomerase activity (Wang, 1985; Brill and Sternglanz, 1988) and by the helical tracking of replication or transcription complexes (Liu and Wang, 1987; Wang and Giaver, 1988; Wu *et al.*, 1988). In prokaryotes, precise regulation of localized DNA tension levels clearly influences the expression of many genes (Drlica, 1984; Pruss and Drlica, 1989); however, in eukaryotes the issue of whether localized torsional tension occurs and its possible function has been controversial (reviewed in Eissenberg *et al.*, 1985; Esposito and Sinden, 1989; Patient and Allan, 1989; Freeman and Garrard, 1992).

A number of assays for detecting torsional tension in DNA have utilized various derivatives of psoralen. These are cell membrane-permeable molecules with a planar, aromatic structure that allows them to intercalate randomly into DNA (Cimino *et al.*, 1985). Upon treatment with 360 nm light, intercalated psoralens mediate crosslinking of opposite DNA strands via formation of covalent bonds at each end of the molecule to adjacent thymines. Because of the particular geometry of the intercalated psoralen within the helix, the most favorable contacts for crosslink formation occur at 5′-TA dinucleotides (Cimino *et al.*, 1985). It has been demonstrated that the rate of psoralen photobinding to DNA is linearly related to its level of negative superhelicity (Sinden *et al.*, 1980). Detection of negative supercoiling *in vivo* can be accomplished by comparing rates of crosslinking in intact cells with those in cells where potential tension has been relaxed by DNA strand nicking (Sinden *et al.*, 1980; Sinden and Ussery, 1992). This is possible because, by definition, unrestrained supercoiling will be relaxed as soon as a swivel (e.g. a strand break) is introduced in the helix. Such an approach also allows a distinction to be drawn between psoralen crosslinking levels (and potential alterations thereof) affected by steric hindrance considerations (e.g. DNA–protein interactions) and those arising from a state of helical twist different from that of fully relaxed DNA (Sinden *et al.*, 1980, 1982). In prokaryotes, measurements averaged globally across the *E.coli* genome have detected unrestrained supercoiling present at a superhelical density (σ) of −0.03 to −0.05 (Sinden *et al.*, 1980). In eukaryotes, similar global assays performed on either HeLa or *Drosophila* cells did not detect torsional tension (Sinden *et al.*, 1980), probably because the majority of eukaryotic chromosomal DNA is wound in nucleosomes (Lacy and Axel, 1975; Garel and Axel, 1976), each of which effectively restrains one negative supercoil (Klug and Lutter, 1981). However, the sensitivity of this assay was not sufficient to detect tension that might be present in a small fraction of the genome, where perturbation of winding associations of DNA with chromosomal proteins in localized regions of chromatin could lead to partial loss of supercoil restraint. Various adaptations of the psoralen probing approach have been used to detect localized torsional tension in bacterial chromosomal DNA (Cook *et al.*, 1989), chloroplast DNA (Thompson and Mosig, 1990; Davies *et al.*, 1991), and constitutively expressed amplified human DNA (Ljungman

and Hanawalt, 1992a). In the present study, we have devised a psoralen crosslinking assay able to search for the existence of tension *in vivo* in precisely defined regions of single copy eukaryotic chromosomal DNA. This method has been utilized to detect localized tension under both transcriptionally silent and active conditions, as well as for mapping the extent of its propagation, in the domain containing the pair of *hsp70* genes at *Drosophila* locus 87A7.

Results

Assay for quantitating specific crosslinks formed in vivo

We have developed a gel-based protocol for quantitating very low levels of psoralen crosslinking localized in specific regions of single copy DNA from eukaryotic cells. Previously reported assays for quantitating psoralen crosslinks in bacterial chromosomal (Cook *et al.*, 1989) and plant chloroplast DNA (Thompson and Mosig, 1990) were not useful in this instance because of sensitivity limitations. In our protocol, the psoralen crosslinked, restriction digested DNA is denatured by glyoxylation, fragments are separated by gel electrophoresis in neutral phosphate buffer (McMaster and Carmichael, 1977), and the gel treated by extended incubation in sodium hydroxide prior to blotting and hybridization (see Materials and methods). Such an approach permits accurate quantitation of both crosslinked (which show a retarded gel mobility) and non-crosslinked fractions of single copy sequences within a complex eukaryotic genome. The coding regions, as well as proximal and distal downstream flanking regions, of the divergent *Drosophila hsp70* genes at locus 87A7 (Figure 1) have been examined for levels of psoralen crosslinking both prior to and after heat induction. Transcriptional activity of these genes rapidly increases to high levels when cultured cells are placed at 37°C (Lindquist, 1986). Transcription-related alterations to the chromatin structure of the *hsp70* genes, including changes in nuclear protein interactions (Wu, 1984; Shuey and Parker, 1986), topoisomerase associations (Fleischmann *et al.*, 1984; Gilmour *et al.*, 1986; Rowe *et al.*, 1986; Kroeger and Rowe, 1989; Udvardy and Schedl, 1991) and nucleosomal character (Wu *et al.*, 1979; Wu, 1980; Udvardy and Schedl, 1984; Karpov *et al.*, 1984; Solomon *et al.*, 1988; Nacheva *et al.*, 1989) have been particularly well documented, thus allowing a rather well-informed interpretation of the data obtained here. In addition to *hsp70*, a coding region fragment of the 18S ribosomal RNA gene (rDNA) has also been analyzed (Figure 1). Because the constitutive rate of rDNA transcription does not change upon thermal elevation (Bell *et al.*, 1988), it provides a control for any non-specific effects of heat shock treatment.

Gene specific crosslinking as a function of heat shock

Figure 2A shows an experiment examining the kinetics of *in vivo* psoralen photobinding to the 18S rDNA under non-heat shock (NHS) conditions. When exposed to increasing light dosages *in vivo*, the fraction of specific fragments crosslinked by psoralen increased (Figure 2A, lanes 2–5) until multiple crosslinks began to accumulate (Figure 2A, lanes 6 and 7). By determining the fraction of crosslinked (XL) versus non-crosslinked (NXL) fragments present in each lane (Figure 2A, lanes 2–5), the number of crosslinks per kilobase (XL/kb) introduced into a DNA fragment by each light dose was determined (see Materials and methods). Similar psoralen crosslinking analyses were performed on the 18S rDNA under heat shock (HS) conditions, and on the *hsp70* coding region under both NHS and HS conditions. Figure 2B summarizes the data obtained. Heat activated *hsp70* genes became crosslinked ~3-fold faster than when they were transcriptionally dormant. Conversely, under either NHS or HS conditions, the constitutively expressed 18S rDNA coding region exhibited an invariant crosslinking rate that lay between the extremes found for the *hsp70* coding region. The data in Figure 2B also show that light dosages of 5–6 kJ/m^2 were in the linear range of response and produced <1 XL/kb in the 1–2 kb restriction fragments tested. Thus, a single light dose of 6 kJ/m^2 was selected for further assays because the values obtained will reflect the

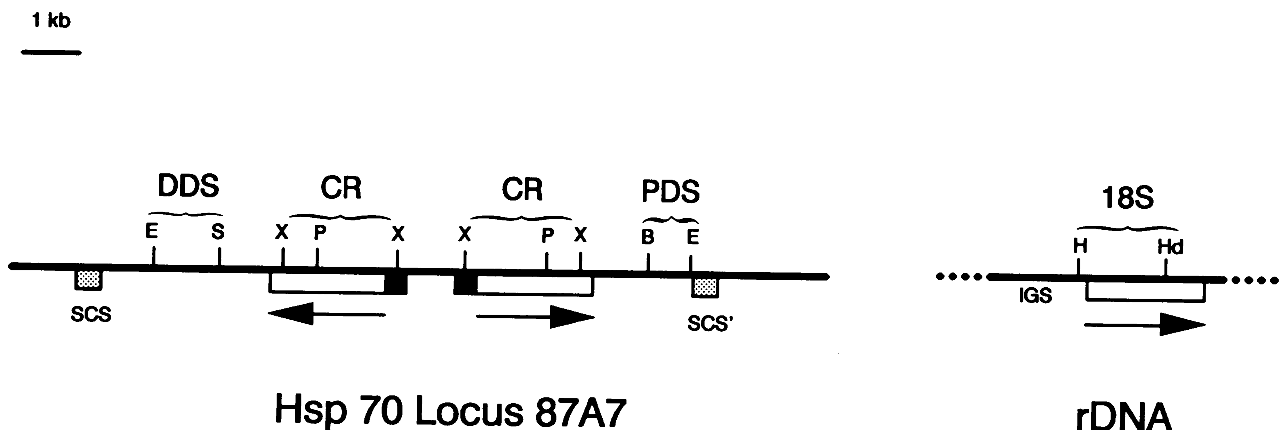

Fig. 1. Maps of the regions examined in this study. At the 87A7 *hsp70* locus on the left the divergently transcribed coding regions (CR) and fragments from the proximal (PDS) and distal (DDS) downstream regions (indicating direction from the centromere) examined are delineated by the restriction sites shown (Goldschmidt-Clermont, 1980; Mason *et al.*, 1982). The open box indicates the transcribed portion of the coding region and the arrows show the direction of transcription. The *Xba*I fragment examined contains the coding region and a small portion of a duplicated 5′ non-coding region (solid box). The locus is flanked by specialized chromatin structures (*scs* and *scs′*) depicted by shaded boxes (Udvardy *et al.*, 1985). A portion of the rDNA locus is shown on the right. The fragment delineated by the restriction sites contains most of the 18S coding region (open box) and a small portion of the intergenic spacer (IGS). This partial map does not show the 5.8S and 28S rDNA which are located immediately downstream of the 18S gene (Long *et al.*, 1981). About 200 copies of the tandemly repeated unit are present in the genome. Restriction enzymes are abbreviated as follows: B, *Bgl*II; E, *Eco*RI; H, *Hae*III; Hd, *Hin*dIII; P, *Pst*I; S, *Sal*I; X, *Xba*I.

relative rates of crosslinking with good precision (Sinden *et al.*, 1980; Sinden and Pettijohn, 1982). The results in Figure 2B also suggest that the crosslinking rates measured were not unduly influenced by the frequency of 5′-TA dinucleotides in the sequences being examined. The *hsp70* coding region fragment has the lowest frequency of 5′-TA of any sequence that we have assayed, yet it exhibits the highest rate of crosslinking when transcriptionally induced. The constitutively active rDNA sequence contains ~10-fold more 5′-TAs per kb, yet has a lower overall rate of crosslinking. Thus, under the conditions of low levels of photo-crosslinking at which the assay is being performed, the 5′-TA frequency is not a major determinant of the rate of crosslinking to specific sequences.

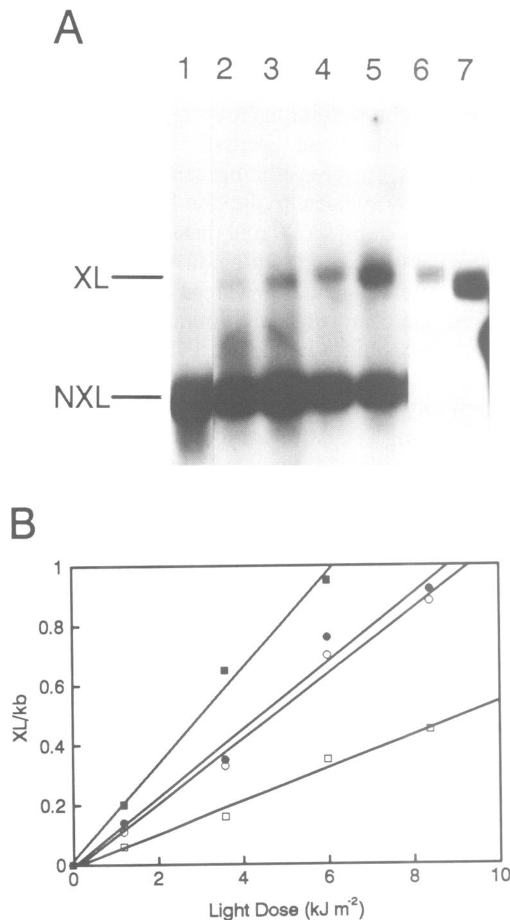

Fig. 2. Characterization of the *in vivo* psoralen crosslinking assay in *Drosophila* chromosomal DNA. (**A**) Agarose gel analysis of psoralen photobinding to specific chromosomal fragments. *Drosophila* Schneider 3 tissue culture cells were equilibrated with psoralen and exposed to 360 nm light for increasing lengths of time to give the following doses in kJ/m^2; 0 (lane 1), 0.6 (lane 2), 1.2 (lane 3), 3.6 (lane 4), 6.0 (lane 5), 12 (lane 6) and 24 (lane 7). DNA was isolated, digested with *Hae*III−*Hin*dIII, glyoxal denatured and analyzed on neutral agarose gels (see Materials and methods). In this experiment, crosslinking rate was determined for the 18S rDNA fragment (Figure 1) under NHS conditions. The NXL and XL fragment bands are designated on the left of the autoradiogram. (**B**) Crosslinking rates of *hsp70* and rDNA coding regions under NHS and HS conditions. Crosslinking rates were determined for the *hsp70* CR (Figure 1) and 18S rDNA fragments in experiments identical to that illustrated in (A). The XL/kb were determined (see Materials and methods) and plotted against the light dose. The points on the graph are indicated as follows: □, NHS-hsp70 CR; ■, HS-hsp70 CR; ○, NHS-18S rDNA; ●, HS-18S rDNA.

Detection of torsional tension in vivo

The observation that the *hsp70* gene is crosslinked much more rapidly when transcribed than when silent does not necessarily reflect an increase in torsional tension engendered by transcription. It is well known from the work of several laboratories that psoralen intercalates highly preferentially in the linker DNA region between nucleosomes (Cech and Pardue, 1977; Sogo *et al.*, 1984). In order to accommodate an intercalator such as psoralen a small amount of base-pair unwinding has to occur, and this is clearly relatively unfavorable on the nucleosome core itself owing to the tight association between DNA and the histone octamer (Morse and Cantor, 1985). However, if such core histone−DNA interactions are perturbed in some way, as has been clearly demonstrated to occur for the nucleosomal array located on the transcribed portion of the *hsp70* genes upon activation (Wu *et al.*, 1979; Nacheva *et al.*, 1989), then access of psoralen to this DNA is likely to be substantially enhanced because the available target size for intercalation has increased. Such alterations in psoralen accessibility caused solely by redistribution of DNA−protein associations have been clearly documented previously (Sinden *et al.*, 1982).

To control for transcription-related alterations in DNA−protein interaction that would affect steric accessibility alone, and to allow an estimate of the contribution that torsional tension makes to the observed rates of crosslinking in cells, we compared crosslinking rates in intact cells with those in cells in which potential torsional tension had been released by DNA nicking immediately prior to crosslinking (Sinden *et al.*, 1980; Sinden and Pettijohn, 1982). This was achieved by culturing cells in medium containing bromodeoxyuridine (BrdUrd) to produce BrdUrd-substituted cellular DNA, which is susceptible to strand breakage upon irradiation with 313 nm light (see Materials and methods). Figure 3 shows representative results from such experiments derived from analysis of restriction fragments encompassing both transcribed (panel A) and proximal downstream flanking (panel B) regions of the *hsp70* genes. Table I summarizes the crosslinking rates per kb calculated from this experiment. The rate of crosslinking in the coding region under NHS conditions was very low compared with the rate upon HS (Figure 3A, compare lanes 2 and 7; Table I; also see Figure 2B). In NHS cells, a nicking light dose of 5 min led to a slight reduction in the *hsp70* coding region psoralen crosslinking rate (Figure 3A, lane 3). A 2-fold reduction relative to intact cells was detectable following a 20 min nicking treatment (Figure 3A, lane 4; also see Table I). In HS cells, a 5 min nicking treatment caused an immediate 2-fold reduction in crosslinking rate relative to intact cells (Figure 3A, compare lanes 7 and 8), and the rate was not reduced upon further nicking (Figure 3A, lane 9; Table I). When psoralen was not added, we did not observe any DNA crosslinking due to exposure of the cells to either the 313 nm wavelength used for nicking or the 360 nm wavelength used for crosslinking (Figure 3A, lanes 5 and 10). The reduction in crosslinking rates in the nicked compared with the intact samples observed in this experiment clearly suggests that torsional tension is present across the coding region in advance of, as well as following, induction of transcription and that its relative level is similar in both cases (discussed in detail below). In notable contrast to the coding region results, similar rates of crosslinking were observed for *hsp70* downstream flanking

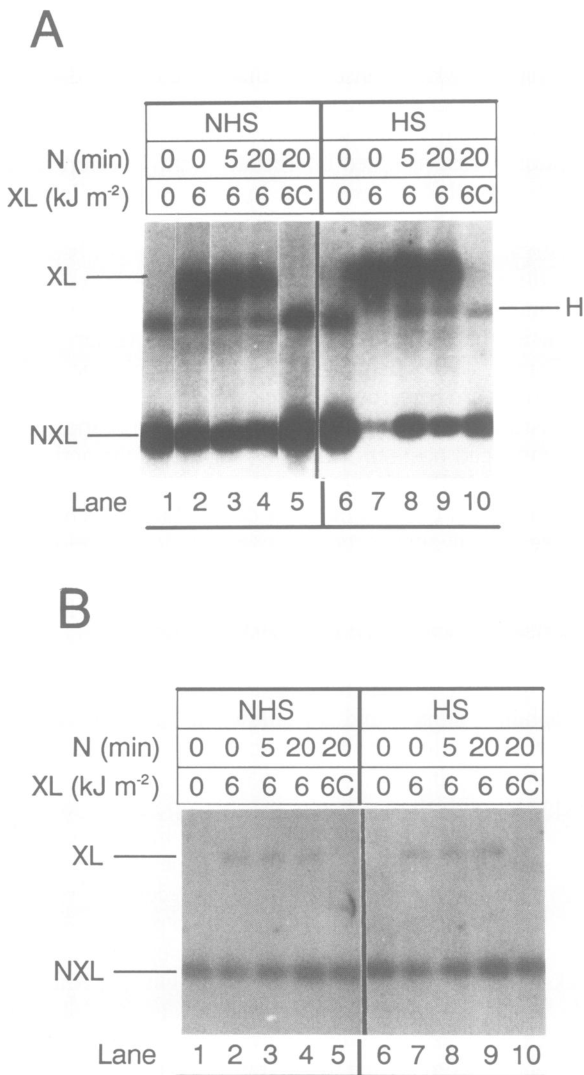

Fig. 3. Psoralen crosslinking *in vivo* in intact and nicked domains of the *hsp70* gene under NHS and HS conditions. Cells with intact or nicked domains were psoralen crosslinked to examine torsional tension. Nicking (N) indicates the time in min that the cells were exposed to 313 nm light. Crosslinking (XL) indicates the dose of 360 nm light in kJ/m^2. The lanes with the 6C label indicate control samples that were both nicked and exposed to crosslinking conditions in the absence of psoralen. The NXL and XL bands produced are indicated on the left hand side. **(A)** Samples were digested with *Xba*I and analyzed as shown above (Figure 2). The *hsp70* CR fragment (Figure 1) is probed in this experiment. The fragment marked H is an additional unique *hsp70* gene fragment from the 87C locus (Mason *et al.*, 1982) that cross-hybridized to the probe. Extended exposures detect the crosslinked fraction for this fragment well above the position of the major crosslinked fraction shown. **(B)** Samples were digested with *Bgl*II–*Eco*RI and probed with the PDS fragment (Figure 1) in this experiment.

regions under both NHS and HS conditions (Figure 3B, compare lanes 2 and 7; Table I). Moreover, under either condition the crosslinking rate was only slightly reduced following the 20 min nicking treatment (Figure 3B, compare lane 2 with lane 4 and lane 7 with lane 9) indicating that substantially less tension was present at this location, particularly under resting conditions prior to transcriptional induction (also see Table I).

Figure 4 summarizes the results of numerous experiments like those shown in Figure 3 performed on the *hsp70* coding

Table I. Crosslinking rates[a] of intact and nicked regions of *hsp70*

	NHS			HS		
	Nicking time (min)			Nicking time (min)		
Region	0	5	20	0	5	20
CR[b]	0.37	0.26	0.19	0.90	0.43	0.39
PDS	0.28	0.23	0.22	0.31	0.28	0.20

[a]These crosslinking rates were determined from the experiments presented in Figure 3 at a light dose of 6 kJ/m^2 as discussed in the text, and are expressed as XL/kb.
[b]Fragment locations and abbreviations are as defined in Figure 1.

region, on *hsp70* distal and proximal downstream flanking regions, and on the 18S rDNA coding region. These data reflect a complex aggregate of both differential accessibility and torsional tension present in chromosomal DNA *in vivo*. To aid in the resolution and discussion of these parameters Figure 4 displays the crosslinking rate per kb for intact domains (solid bar) adjacent to the rate for the same domain when relaxed by nicking for 20 min (hatched bar). The height of the solid bars for each region investigated reflects a composite of differential psoralen crosslinking derived from both the presence of unrestrained torsional tension and the overall helix accessibility in intact cells, while the height of hatched bars necessarily reflects that component derived from helix accessibility alone since any potential tension has been released by nicking. Thus, the ratio of the crosslinking rate in intact (solid) versus relaxed (hatched) chromosomal domains (shown at the bottom of Figure 4 as $R_{I/N}$) reflects DNA tension levels in a given fragment, and the relative ratio comparing one region with another reflects differential partitioning of this tension within a given domain.

The critical basis for interpreting a reduction in crosslinking rates upon nicking as reflecting a release of incipient unrestrained supercoiling, equilibrated as torsional tension, relies on the expectation that the nicking process does not alter nuclear protein binding or association with DNA in a manner that would cause greatly reduced steric accessibility of psoralen to the helix. Previous studies addressing global tension and accessibility levels in viral (Sinden and Pettijohn, 1982; Sinden *et al.*, 1982), bacterial and eukaryotic (Sinden *et al.*, 1980) genomes have demonstrated that nicking procedures as used here do not appear to induce a non-specific agglomeration ('crashing') of proteins on to DNA. However, in order to address the possibility that such 'crashing' effects could be occurring at specific loci and thus be an artifactual cause of the observed reduction in psoralen crosslinking, *in vivo* DNA–protein crosslinking experiments with formaldehyde were performed according to the procedures of Solomon *et al.* (1988) on both intact cells and cells exposed to nicking conditions identical to those used in the psoralen assay. This allowed an assessment of the effect of nicking on the level of protein associations with the particular DNA fragments examined in this study. Figure 5 shows agarose gel shift analyses of crosslinked DNA–protein samples derived from these experiments. In intact cells a retardation in migration of both *hsp70* and rDNA fragments is evident when comparing control samples with pronase-resistant, formaldehyde treated samples (compare lanes 1 and 2 for

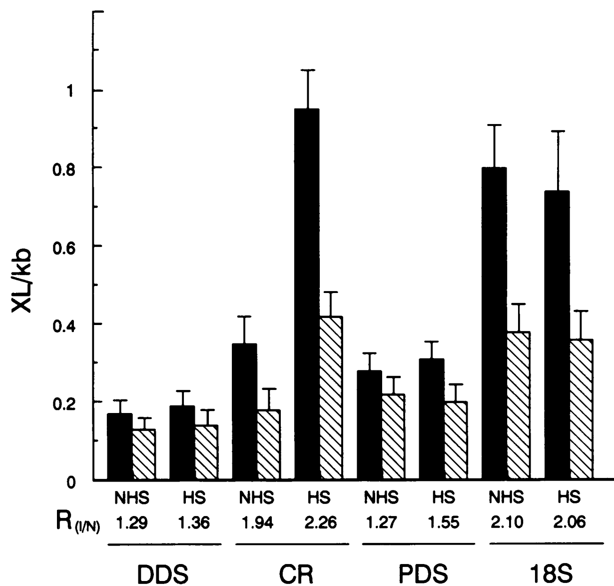

Fig. 4. Schematic comparison of the crosslinking rates in intact and nicked samples from NHS and HS cells. The rate of crosslinking per kilobase is plotted on the vertical axis. The solid bars show the rate of crosslinking in intact cells. The rate for samples nicked for 20 min is depicted by the hatched bars. Each of the bars represents the average of six crosslinking rates determined from three independent cell preparations, each of which was analyzed twice. The standard deviation for each of the values is shown by the error bars. The ratio of the mean crosslinking rates calculated for intact/nicked domains ($R_{I/N}$) is presented for each of the four restriction fragments examined. All abbreviations are as defined in Figure 1.

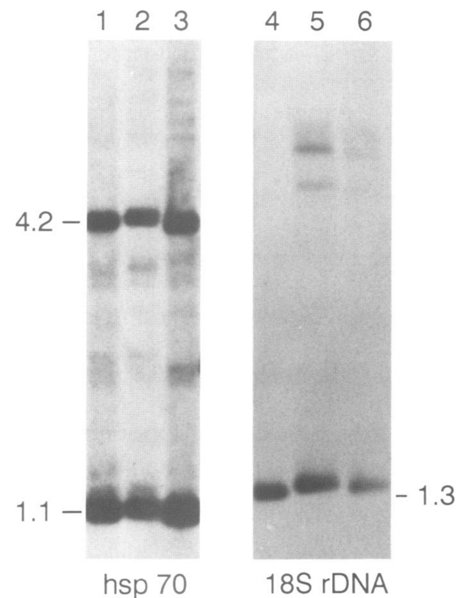

Fig. 5. Analysis of DNA–protein interactions *in vivo* by formaldehyde crosslinking in intact and nicked chromosomal domains. Purified pronase-resistant nucleoprotein complexes were digested extensively with the restriction enzyme of choice and analyzed by blot/hybridization following TAE agarose gel electrophoresis. The samples analyzed are purified DNA (lanes 1 and 4), formaldehyde crosslinked DNA–protein fragments from intact cells (lanes 2 and 5) and formaldehyde crosslinked DNA–protein fragments from cells subjected to UV nicking (lanes 3 and 6). The *hsp70* regions (lanes 1–3) were examined in *Pst*I digests (see Figure 1) and probed with an *Xba*I–*Pst*I fragment from the 5′ end of the coding region. Thus, the 4.2 kb band is derived from locus 87A7 (see Figure 1). The 1.1 kb band detected in this experiment is derived from *hsp70* genes at locus 87C1 which have an additional *Pst*I site adjacent to the *Xba*I site at the 5′ end of the coding region. The rDNA region (lanes 4–6) examined is the same as in Figure 2A. The marker sizes shown to the left and right of the panels refer to the size of the purified DNAs in lanes 1 and 4 respectively.

hsp70, and lanes 4 and 5 for rDNA). In formaldehyde treated samples from cells exposed to DNA nicking conditions a shift in migration compared with purified DNA is still evident (compare lanes 1 and 3 for *hsp70*, and lanes 4 and 6 for rDNA), but the shift is, if anything, slightly reduced relative to that of the formaldehyde treated samples from intact cells (compare lanes 2 and 3 for *hsp70*, and lanes 5 and 6 for rDNA). If a protein 'crashing' artifact were occurring upon nicking a further retardation in the migration of *hsp70* or rDNA fragments would have been expected owing to increased opportunities for DNA–protein crosslink formation. We conclude from these data and other controls (see Discussion) that there is no reasonable interpretation for the dramatic reduction of psoralen crosslinking upon nicking other than the release of a pre-existing level of torsional tension from selected regions of the *Drosophila* genome.

Discussion

A stable microdomain of torsional tension

Several conclusions can be drawn concerning the existence of torsional tension *in vivo* as well as the relative accessibility of the various *Drosophila* chromosomal regions that we have studied. Even prior to heat induced activation, the *hsp70* coding region is in a state of torsional tension as indicated by the 2-fold reduction in crosslinking rate observed after nicking (CR in Figure 4). The flanking regions show a slight reduction in psoralen binding (comparing intact and nicked samples) suggesting that only very low levels of torsional tension are constitutively present at these downstream locations (DDS and PDS in Figure 4). Thus, it appears that we are observing the existence of a region of stably unrestrained supercoiling, with boundaries that are located somewhere between the end of the *hsp70* coding region and the downstream flanking sequences. In this sense, the experiments have defined an independent topological domain. Following heat induced activation, the *hsp70* coding region becomes almost 3-fold more accessible to psoralen, undoubtedly a reflection of the large nucleosomal perturbation associated with active transcription (Wu *et al.*, 1979; Wu, 1980; Karpov *et al.*, 1984; Nacheva *et al.*, 1989), but it again shows the same 2-fold reduction in crosslinking rate upon nicking (CR in Figure 4). Such results lead to the conclusion that this large increase in psoralen accessibility upon heat shock is due to the known major nucleosomal perturbation that occurs upon transcription (i.e. the target size for psoralen intercalation increases from that due to linker DNA alone to that of linker plus formerly core-associated DNA): there is, however, no corresponding change in overall tension levels as a result of gene activation. Moreover, if there were a tension-related component to the instability of the nucleosomal array upon transcriptional induction (e.g. a supercoil-driven unfolding of the nucleosomes) then it would be anticipated that upon nicking the nucleosomes would refold, and the level of psoralen crosslinking in nicked heat shocked cells would be similar

to that in nicked control cells. This is clearly not the case. Little change in steric accessibility to psoralen in the 3′ flanking regions of *hsp70* is caused by heat shock (DDS and PDS in Figure 4), consistent with the apparent stability of nucleosome structure in these particular sequences upon gene activation (Udvardy *et al.*, 1985). However, the $R_{I/N}$ does increase slightly following heat shock, suggesting that the flanking regions may be experiencing somewhat more tension, perhaps as a consequence of tracking RNA polymerases.

The sum of the data indicated that heterogeneous torsional tension levels exist within the 87A7 structural domain regardless of transcriptional activity. This locus is flanked by sequences (Figure 1) termed specialized chromatin structures (*scs*), first identified by nuclease sensitivity assays (Udvardy *et al.*, 1985) and recently shown to act in genetic tests as effective insulators of chromosomal position effects (Kellum and Schedl, 1991, 1992). The level of constitutive tension at 87A7 is, however, significantly reduced immediately downstream of the *hsp70* coding region, well before the *scs* sequences are encountered. If the *scs* sequences are viewed as some form of boundary for the definition of a structural or genetic domain at locus 87A7 (only a supposition at this point), our data indicate that such a domain does not correspond physically to the more limited topological domain defined here. Interestingly, the extent of propagation of high levels of torsional tension falls within boundaries apparently delineated by a recently described *Drosophila* chromatin binding protein named B52 that associates with the downstream borders of actively transcribed regions of 87A7 (Champlin *et al.*, 1991). However, this protein is not present under conditions of transcriptional inactivity; therefore, our detection of a limited topological domain prior to gene activation appears to preclude a role for B52 in delineating tension boundaries. It is certainly not necessary to hypothesize the existence of a sequence-specific boundary to explain our observations. It could easily be the case that the tension across the *hsp70* genes is confined by the presence on flanking DNA regions of nucleosomes that, by virtue of tight binding to DNA, do not allow further transmission of tension. Certainly this is a property expected of nucleosomal-bound DNA based on earlier *in vitro* observations (Morse and Cantor, 1985). At this time we have no knowledge of whether the tension present across the inactive *hsp70* genes is confined to nucleosomal linkers or is equilibrated through the core-associated DNA.

Minimal differences in the accessibility of the 18S rDNA occur in response to heat shock (18S in Figure 4), in congruence with the reported lack of effect of thermal elevation on transcription of these genes (Bell *et al.*, 1988). However, 2-fold reductions in crosslinking rates upon nicking are again evident, irrespective of heat shock, reflecting the presence of constitutive torsional tension at this location also (Figure 4). Thus, both of the coding regions examined in our study contain localized torsional tension at significantly higher levels than in non-transcribed regions. A recent study of the 5′ end of the coding region of the amplified dihydrofolate reductase genes and the 18S rDNA of human cells (both constitutively expressed) has reported a similar reduction in psoralen crosslinking levels upon nicking (Ljungman and Hanawalt, 1992a). Thus, localized torsional tension appears to be detectable in several eukaryotic genes. It is important to remember that previous studies of unrestrained supercoiling averaged globally across either *Drosophila* (Sinden *et al.*, 1980) or human (Sinden *et al.*, 1980; Ljungman and Hanawalt, 1992a) genomes failed to detect any deviation from the situation expected for relaxed, or at least fully restrained, chromosomal DNA. The data we have obtained are not inconsistent with this observation given that only a very small fraction of the genome is (or has the potential to be) actively transcribed. We are currently assaying other non-transcribed or transcribed regions of the *Drosophila* genome *in vivo* to confirm the generality of the observation that non-transcribed sequences immediately flanking active genes exist under zero (or very low) levels of tension.

Previous analyses of psoralen crosslinking rates *in vitro* in intact topological isomers at defined levels of superhelical density (σ), suggested that a 2-fold reduction in psoralen crosslinking rates upon relaxation corresponds to a σ equal to -0.08, while a 1.2- to 1.5-fold reduction occurs at a σ of -0.01 to -0.02 (Sinden *et al.*, 1980). Based on a 1.7-fold reduction in crosslinking rates upon nicking, the effective σ of *E.coli* chromosomal DNA was estimated to be -0.05, a figure that correlated well with earlier estimates (discussed in Sinden *et al.*, 1980) but at the high end of more recent determinations made from a variety of *in vivo* assays for supercoiling in bacterial plasmids (reviewed by Drlica *et al.*, 1992). Thus, in principle we could be detecting magnitudes of localized effective σ in the coding regions of *hsp70* and rDNA similar to those found in the *E.coli* chromosome, although the actual σ remains to be measured by a more quantitative approach. In contrast, the flanking regions of the *hsp70* genes may have a 4- to 8-fold lower effective σ than the coding region depending on gene activity. Experiments attempting to estimate precisely the *in vivo* level of σ present at these locations are currently underway utilizing strategies applied previously in prokaryotic organisms (Kochel and Sinden, 1988; Dröge and Nordheim, 1991; Zheng *et al.*, 1991).

Applicability of the psoralen assay for detecting in vivo tension

As discussed above in Results, alternative explanations for the dramatic reduction in psoralen crosslinking that occurs in selected genomic locations after nicking of chromosomal domains have been considered and tested. The results provide no evidence for a non-specific effect of nicking on overall steric accessibility of the DNA helix to psoralen. Thus, the reduction in psoralen photobinding that we observe upon nicking is detecting the presence of unrestrained supercoiling. We also performed extensive control experiments to address whether rates of psoralen crosslinking are influenced by the lowered temperature conditions at which the assay is performed. We did not observe significant differences in psoralen crosslinking levels when the 360 nm light treatment was performed at 25°C immediately following harvesting compared with cells that were harvested, brought to 0°C, and left on ice for up to 30 min prior to crosslinking. Similar crosslinking rates were also observed for samples that were heat shocked and immediately crosslinked compared with those kept on ice for up to 30 min (unpublished data). These controls suggest that there is no immediately detectable effect of temperature changes on the levels of negative DNA supercoiling. Based on studies that have quantitated the

change in helical twist of DNA as a function of temperature (Goldstein and Drlica, 1984), we estimate that, at the upper limit, 1 kb of completely protein-free DNA transferred from 37 to 0°C in the absence of topoisomerase activity could have one negative supercoil introduced. In eukaryotic chromatin, this level of change should be substantially less by virtue of association of the DNA with nucleosomes (reported to restrain effectively temperature-induced changes in twist; Morse and Cantor, 1985) or other chromosomal proteins. Thus, any negative supercoiling that might be introduced by lowering temperature was not detectable in control experiments and is likely to be a negligible component of the levels we actually observe.

A particularly interesting feature of the *in vivo* nicking experiments (Table I) is that the sensitivity of the actively transcribing *hsp70* domain to nicking (minimum value reached in 5 min) was substantially greater than in the transcriptionally inactive case (where it took 20 min to reach the same minimum). One interpretation of this result is that the size of the topological microdomain increased substantially upon heat shock. We do not favor this possibility, however, and prefer an explanation based on the chemistry of the nicking reaction, which involves UV-induced free radical production mediated by BrdUrd. Recent experiments have shown that in purified nuclei the DNA in transcriptionally active domains was far more susceptible to oxidative damage by free radicals than was that in inactive domains, suggesting that the difference in damage sustained was due to differences in chromatin condensation (Ljungman and Hanawalt, 1992b). Our observations are consistent with this type of mechanism operating *in vivo* in intact cells.

The relatively constant crosslinking frequencies observed for the *hsp70* downstream, as well as the 18S rDNA coding, fragments under both NHS and HS conditions (Figure 4) provide an important internal control for the validity of this assay. The data show that the major differences in crosslinking rates across the *hsp70* coding region are not due to psoralen delivery differences mediated by differential membrane permeability caused either by the elevated temperatures used to induce heat shock gene transcription or by the *in vivo* nicking treatment.

In summary then, we believe that when performed with the appropriate controls (as we have been particularly careful to do), this psoralen crosslinking approach provides an appropriate and valid method for detecting torsional tension in eukaryotic chromatin *in vivo*.

Biological relevance and significance

The existence of a stable, constitutive microdomain of torsional tension in the *hsp70* genes is a rather unexpected finding and is therefore of particular interest. An important question is obviously what is/are the process(es) leading to its establishment and maintenance? Up to this time, there has been no demonstration of an enzymatic activity in *Drosophila* (or any other eukaryote for that matter) that is able, like prokaryotic DNA gyrase, to introduce negative supercoils into DNA (discussed in Clarke and Wolffe, 1991; recently reviewed in Freeman and Garrard, 1992). Establishment by this means thus appears to be a remote possibility. However, there are, as noted in the Introduction, other mechanisms by which negative supercoiling could be generated. For example, release of restrained DNA supercoils by loss of nucleosomes (see Esposito and Sinden, 1989; Freeman and Garrard, 1992), or via altered associations with high mobility group proteins (Ridsdale *et al.*, 1990) and/or other components of the chromatin fiber in the immediate vicinity of the *hsp70* genes could introduce negative supercoils. In this context, it may be relevant that both the promoter and transcription initiation regions of uninduced *hsp70* genes exist in a DNase I hypersensitive configuration (Wu, 1980), and therefore are considered to be nucleosome free. We are currently in the process of mapping the precise boundaries of the supercoiled domain in order to determine if there are significant, nick-responsive, hot-spots of psoralen cross-linking through the body of the gene or in its promoter. As a further possibility it has recently been reported that acetylated histones as constituents of nucleosomes restrain only 80% of the supercoils of their non-acetylated counterparts (Norton *et al.*, 1989, 1990). Since acetylated nucleosomes are strongly correlated with active chromatin (Hebbes *et al.*, 1988; Tazi and Bird, 1990), it is possible that the transcriptionally competent *hsp70* genes are enriched in nucleosomes of this type, and thus exist in a region of partially unrestrained supercoils. Immunofluorescence analysis has demonstrated the presence of various forms of acetylated histone on *Drosophila* polytene chromosomes (Turner *et al.*, 1992). Further analysis of their relative distribution at the heat shock loci will be of some interest.

A particularly intriguing feature of the *hsp70* genes is that they have been found to exist in a 'transcriptionally poised' configuration. Apparently, not only is a molecule of RNA polymerase II present on each gene in advance of activation, but it is transcriptionally engaged and elongationally stalled, having synthesized a short transcript up to 25 bases long (Rougvie and Lis, 1988; O'Brien and Lis, 1991). It is an intriguing possibility that the existence of the unrestrained tension that we detect here is related to the presence of this paused polymerase transcription complex. Loss, or perturbation, of nucleosomes might either accompany the establishment of this configuration, or indeed be required to help promote its assembly. The importance of underwound DNA in promoting efficient assembly of stable eukaryotic RNA polymerase II initiation complexes has been suggested by a number of *in vitro* experiments (Tabuchi and Hirose, 1988; Mizutani *et al.*, 1991). Furthermore, recent *in vivo* experimental approaches have documented the presence of torsional tension during the assembly of competent RNA polymerase II transcription complexes within chromatin (Leonard and Patient, 1991), and the enhancement of rates of RNA polymerase I transcriptional initiation by negative supercoiling (Schultz *et al.*, 1992). We are certainly very interested in the potential correlation between negative superhelicity and engaged RNA polymerase activity suggested by our observations and are currently studying other genes known to lack such complexes in the cell type under study. This is not necessarily just a trivial exercise of looking at silent genes—the elongational blocking of transcriptionally engaged polymerases may be a much more common method of regulating transcription than heretofore supposed (Rougvie and Lis, 1990; Spencer and Groudine, 1990). Consequently, it will be important to document the absence of this 'engaged but blocked' feature in future crosslinking assays of inactive genes.

Given the existence of a limited domain of supercoiling in advance of transcription why is it not released by the action

of topoisomerases within the nucleus? Up to this time there has been no demonstration of topoisomerase I activity in or around the *hsp70* genes prior to transcription (Fleischmann *et al.*, 1984; Kroeger and Rowe, 1989). Studies with inhibitors of topoisomerase II have revealed cutting within the *hsp70* chromatin (Rowe *et al.*, 1986; Kroeger and Rowe, 1992), but no evidence for unregulated activity of the enzyme *in vivo* has yet been obtained. Our data suggest, at the very least, that the relaxing action of such enzymes must be strictly regulated *in vivo*. Moreover, the data indicate that the domain of supercoiling (and its absolute level) is maintained even in the face of active transcription, a situation in which large amounts of topoisomerase I are clearly recruited to the transcribed sequences (Fleischmann *et al.*, 1984; Gilmour *et al.*, 1986; Kroeger and Rowe, 1989), probably in close association with RNA polymerase II molecules (Stewart *et al.*, 1990). Thus, any local tension generated by tracking of the RNA polymerase complex (as anticipated by the model of Liu and Wang, 1987) is presumably rapidly relieved in the purely local frame of reference, so that levels in excess of that constitutively present do not accumulate across the transcribed region. Our observation that the net level of supercoiling equilibrated across the *hsp70* domain is maintained in the face of transcription indicates once again that the relaxing activity of topoisomerase (in this case both topoisomerases I and II) is not ubiquitously expressed throughout the nucleus but must be spatially and specifically regulated by as yet unknown mechanisms. In the context of topoisomerase I activity within eukaryotic nuclei, our observations strongly support a model of quantitative and temporal linkage to RNA polymerase II activity that was recently proposed on the basis of an extensive series of studies on transcription of the c-*fos* gene (Stewart *et al.*, 1990). Given the presence of stalled polymerases on transcriptionally inactive *hsp70* genes and the co-existence of a constitutive level of tension it would be reasonable at this point to postulate that topoisomerase I activity is expressed only in the context of an actively elongating polymerase, the enzyme perhaps being recruited as part of an elongation factor complex.

Materials and methods

Cell culture

Schneider line 3 cells were grown in Schneider's *Drosophila* medium supplemented with 7% fetal calf serum and 0.5% bactopeptone containing 50 μM BrdUrd. The BrdUrd was included in all cell preparations because its incorporation into the DNA is necessary for producing the nicked controls (see below). We have not observed significant differences in psoralen crosslinking rates between cells grown with and without BrdUrd (Sinden and Pettijohn, 1982; unpublished data).

Nicking domains in vivo

In a given experiment on NHS or HS cells the nicking treatment was performed on one half of the cells rapidly chilled (and kept) on ice while irradiating with 313 nm light from a high-pressure mercury lamp through a K_2CrO_4/NaOH filter (Sinden and Pettijohn, 1982). Following this, both untreated and treated cells were immediately carried through the psoralen crosslinking and DNA purification protocol (see below). The nicking doses chosen were determined by examining the kinetics of nicking for specific genes on 0.6% agarose gels. We estimated that the initial nicking time of 5 min created one nick per 40 kb while the 20 min dose produced one nick per 15–20 kb in the regions examined in this study.

Psoralen photobinding

Cells grown to a density of $3-5\times10^6$ cells/ml were either used immediately (NHS) or incubated at 37°C for 30 min (HS), and then subjected to crosslinking in the intact or nicked (described above) condition. Cells were rapidly chilled on ice (or kept on ice if already nicked), and psoralen (4,5′,8-trimethylpsoralen) was added to a final concentration of 0.2 μg/ml in medium and allowed to equilibrate for 5 min (Sinden *et al.*, 1980). The cells were poured into a Petri dish and exposed for varying lengths of time (generally 0–10 min) to 360 nm light at a dose of 1.2 kJ/m^2/min delivered by two General Electric F20T12/BLB bulbs. The DNA was then purified using standard procedures (Cartwright and Elgin, 1986). No differences in the crosslinking data were obtained when 360 nm irradiations were performed at either 25°C or (where relevant) 37°C rather than at 0°C.

Gel analysis of photocrosslinked DNA

Purified DNA was digested to completion with restriction enzymes that produce fragments containing the regions of interest. At the very low levels of photobinding used we have not observed any inhibition of digestion caused by crosslinks. Following phenol/chloroform extraction, reprecipitation and resuspension in water, DNA samples were denatured by boiling for 2 min and treating with glyoxal (McMaster and Carmichael, 1977). Glyoxylated NXL and XL fragments were then separated by electrophoresis on 1% neutral agarose gels cast and run in 10 mM sodium phosphate (pH 7.0) as previously described (McMaster and Carmichael, 1977). After electrophoresis gels were incubated in standard denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 1 h. Empirical observation showed that this incubation of the gel containing glyoxal denatured DNA was essential for efficient probe hybridization to the psoralen crosslinked fraction. DNA fragments were transferred to nylon membranes (MSI, Magna-NT) in 0.4 M NaOH (Reed and Mann, 1985), and membranes were hybridized to ^{32}P-labeled gel purified fragments produced by random priming (Feinberg and Vogelstein, 1983), then washed and exposed to a phosphor screen. The *hsp70* fragments (see Figure 1) used as probes were purified from plasmid 122 (Goldschmidt-Clermont, 1980), and the rDNA fragment was isolated from plasmid DmrY22 (Dawid *et al.*, 1978). Quantitation of NXL and XL fractions was performed using a Molecular Dynamics Phosphorimager and ImageQuant™ software. Autoradiograms were also made for illustrative purposes using preflashed Kodak XAR-5 film.

Analysis of DNA–protein interactions by formaldehyde crosslinking

Tissue culture cells with domains either intact or nicked by photolysis for 20 min, as described above, were crosslinked with 1% formaldehyde for 8 min at 4°C in growth medium, and subjected to pronase digestion as previously described in detail (Solomon *et al.*, 1988). Following two precipitations of the nucleoprotein complexes with 2 M ammonium acetate and isopropanol, samples were resuspended in 1 mM Na_2EDTA, 10 mM Tris–HCl (pH 8.0) and digested extensively with the appropriate restriction enzyme. Gel shifts were detected by separation on TAE agarose gels followed by blot/hybridization analysis. For analysis of *hsp70*, *Pst*I digests were probed with an *Xba*I–*Pst*I fragment from the 5′ end of the *hsp70* coding region. The rDNA was analyzed as described above.

Data analysis

The fraction of DNA crosslinked (F_x) was determined by dividing the integrated area of the crosslinked band by the sum of the integrated areas of the non-crosslinked and crosslinked bands. Assuming a Poisson distribution the mean number of crosslinks per fragment (XL/Fg) was calculated using the formula [$XL/Fg = -\ln(1-F_x)$]. The crosslinks per kb were then calculated.

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