Analysis of Topoisomerase I and II Cleavage Sites on the *Drosophila* Actin and Hsp70 Heat Shock Genes†

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ABSTRACT: We have compared topoisomerase I and II cleavage sites on the actin 5C and 57A genes and the hsp70 genes in *Drosophila* K_c cells using the inhibitors camptothecin (topoisomerase I specific) and VM-26 (topoisomerase II specific) to assess the role of these enzymes in transcriptional regulation. Topoisomerase I cleavage sites were localized to the transcribed regions of the actin 5C and hsp70 genes and were present only when these genes were active. The actin 57A gene, shown previously to be inactive in K_c cells, had no detectable topoisomerase I cleavage sites. In contrast to topoisomerase I, topoisomerase II cleavage sites could be detected on transcriptionally active and inactive actin and hsp70 DNA sequences. Topoisomerase II cleavage sites on the inactive hsp70 gene were primarily localized to the very 5′ end of the transcribed region of the gene. However, upon heat-induced activation of hsp70 transcription, topoisomerase II cleavage rapidly shifted from the 5′ to the 3′ end of the gene. Then, during the shutdown of hsp70 expression, there was a gradual reappearance of topoisomerase II cleavage at the 5′ end of the gene that temporally correlated with the repression of hsp70 transcription. There was a similar preferential association of topoisomerase II with the 5′ ends of transcriptionally repressed actin 5C and 57A genes. These results demonstrate that there are marked differences in how topoisomerases I and II interact with transcriptionally active and inactive regions of chromatin. In addition, we have identified an unusual type of topoisomerase II binding site that is preferentially associated with the 5′ ends of inactive hsp70 and actin genes, suggesting that this enzyme may facilitate changes in chromatin structure that are associated with repression of gene transcription.

DNA topoisomerases are enzymes that change the topological structure of DNA by breaking and rejoining the DNA phosphodiester backbone (Liu, 1989; Maxwell & Gellert, 1986; Vosberg, 1985; Wang, 1987). In eukaryotic organisms, type I and II topoisomerases catalyze the relaxation of both positive and negative supercoils from DNA by breaking and rejoining one or both strands of the DNA double helix, respectively (Liu, 1989; Maxwell & Gellert, 1986; Vosberg, 1985; Wang, 1987). Genetic and biochemical data suggest that topoisomerase-mediated changes in DNA topology are important in eukaryotic gene expression, DNA replication, and chromosome segregation [reviewed in Sternglanz (1989) and Hsieh (1990)].

Topoisomerases I and II have been shown to be the targets of several important classes of antitumor drugs [reviewed in Liu (1989) and Drlica and Franco (1988)]. DNA topoisomerase I is specifically inhibited by the drug camptothecin whereas topoisomerase II is inhibited by anthracycline, ellipticine, amsacrine, and epipodophyllotoxin type drugs. these drugs inhibit topoisomerase function by stabilizing a covalent enzyme–DNA intermediate termed the cleavable complex (Liu, 1989). Treatment of this complex with a protein denaturant results in either single-stranded (topoisomerase I) or double-stranded (topoisomerase II) protein-linked DNA breaks. Drug-induced topoisomerase breaks have been mapped on cellular chromosomes from a variety of organisms to ascertain how these enzymes might regulate chromatin structure and function. Camptothecin-induced topoisomerase I cleavage sites have been mapped on the heat shock genes in *Drosophila*, the rat tyrosine aminotransferase gene, the human c-*fos* gene, and the ribosomal genes in human, *Xenopus laevis*, and *Dictyostelium* cells (Gilmour & Elgin, 1987; Kroeger & Rowe, 1989; Stewart & Schutz, 1987; Stewart et al., 1990; Zhang et al., 1988; Culotta & Sollner-Webb, 1988; Ness et al., 1988). Cleavage sites have also been mapped on SV40 virus DNA in infected monkey kidney cells (Porter & Champoux, 1989). The results from these studies showed that topoisomerase I cleavage was localized to the transcribed region of active, but not inactive, genes, suggesting that this enzyme was involved in ongoing gene expression. These results are consistent with immunofluorescence and photo-cross-linking studies in *Drosophila* that also indicate a preferential association of topoisomerase I with actively transcribed sequences in DNA (Fleishchmann et al., 1984; Gilmour et al., 1986).

Drug-induced topoisomerase II cleavage sites have been characterized in vivo on the *Drosophila* heat shock protein 70 (hsp70)[1] gene (Rowe et al., 1986), the human c-*myc* protooncogene (Riou et al., 1986, 1989), the chicken β-globin genes (Muller & Mehta, 1988; Reitman & Felsenfeld, 1990), and on SV40 and adenovirus chromatin in virally infected cells (Chen et al., 1984; Yang et al., 1985a,b; Schaack et al., 1990). In contrast to topoisomerase I, topoisomerase II cleavage sites were localized near or within DNase I hypersensitive regions at the 5′ and/or 3′ ends of genes. Cleavage in these regions was often but not always associated with ongoing transcription. In the case of the chicken globin genes, there appeared to be two types of topoisomerase II DNA binding sites (Reitman

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[1] Abbreviations: hsp, heat shock protein; SSPE, 180 mM NaCl/10 mM sodium phosphate (pH 7.7)/1 mM EDTA; SSC, 0.15 M NaCl/15 mM sodium citrate, pH 7.0; Act D, actinomycin D; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate.

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& Felsenfeld, 1990). The first type of topoisomerase II DNA binding site was temporally coupled to the activation of the globin gene transcription during erythroid cell development, suggesting that topoisomerase II may function to relieve topological stress generated during globin gene transcription. However, the second type of topoisomerase II binding site was unaffected by changes in globin gene transcription and was present throughout erythroid development. It was suggested that binding of topoisomerase II to this second type of site may play a general role in the organization of chromosomal loops in the nucleus (Reitman & Felsenfeld, 1990).

With the exception of the adenovirus study (Schaack et al., 1990), there has been no attempt to directly compare topoisomerase I and II binding sites on transcriptionally active and inactive DNA sequences. In this regard, we have systematically investigated topoisomerase I and II binding sites on transcriptionally active and inactive actin and hsp70 gene sequences in *Drosophila* K_c cells using the inhibitors camptothecin and VM-26. Our results indicate that there are significant differences in how these two enzymes interact with chromatin during transcription. These studies also identify a new type of topoisomerase II binding site that is preferentially associated with the 5′ ends of inactive genes. The presence of topoisomerase II activity at this novel type of binding site may play a role in facilitating changes in chromatin structure that are associated with the repression of gene transcription.

Materials and Methods

Enzymes, Nucleic Acids, and Drugs. Restriction endonucleases were obtained from Boehringer-Mannheim (Indianapolis, IN) and New England Biolabs Inc. (Beverly, MA). Materials necessary for nick-translation were purchased from BRL (Gaithersburg, MD). The sodium form of camptothecin was obtained from the National Cancer Institute, and epipodophyllotoxin VM-26 was kindly provided by the Bristol-Myers Co. These drugs were prepared as 100 mM stocks in DMSO and stored frozen at −20 °C until use. The *Drosophila* hsp70 DNA clone pMR4 is a subclone that contains the 610 bp *Bam*HI–*Xba*I fragment of pPW229 (Corces et al., 1978) cloned into the *Bam*HI site of the vector pGEM 2 (Promega Biotech, Madison, WI). The *Drosophila* actin 5C DNA clone p5CPS is a subclone of the 3.6-kb *Eco*RI–*Sal*I fragment from pDmA2 (Fyrberg et al., 1981) that contains the 590 bp *Pst*I–*Sal*I fragment in pGEM 1. The *Drosophila* actin 57A subclone p57APH is a subclone of the 1.6-kb *Bam*HI–*Hin*dIII fragment from pDmA4 and consists of a 300 bp *Pst*I–*Hin*dIII fragment in pGEM 1.

Cell Culture. Drosophila K_c cells were obtained from Dr. Neil Osheroff (Vanderbilt University). The cells were maintained at 23 °C in D-22 medium supplemented with penicillin and streptomycin as described previously (Schneider & Blumenthal, 1978). Heat shock treatments were performed in a water bath at 33 or 37 °C.

Isolation and Analysis of Cellular DNA. Cellular DNA was purified from cultured *Drosophila* K_c cells as described before (Kroeger & Rowe, 1989). Samples (25 μL) containing 10 μg of restricted DNA were combined with 3 μL of a loading cocktail (50% sucrose, 0.05% bromophenol blue, 0.05% xylene cyanol, and 0.1% SDS) and electrophoresed in a 1.4% horizontal agarose gel containing 0.5× TBE (45 mM Tris base, 45 mM boric acid, and 1 mM EDTA, pH 8.3) for 16 h at 70 V. DNA was transferred from the agarose gels onto nitrocellulose filter paper (BA 85, Schleicher & Schuell, Keene, NH) as previously described (Southern, 1975; Maniatis et al., 1982). The filters were then preincubated in DNA hybridization buffer (5× SSPE, 0.1% SDS, and 0.25% nonfat dry milk) at 67 °C. After 4 h, the filters were placed in fresh DNA hybridization buffer containing [^{32}P]-labeled probe and incubated at 67 °C for an additional 20 h. The filters were then washed in 0.1 M potassium phosphate, pH 7.0, for 30 min and then in 1× SSC/0.5% SDS for an additional 30 min at room temperature. A final wash was done in 0.1× SSC/0.5% SDS at 65 °C for 30 min.

Isolation and Analysis of Cellular RNA. Total cellular RNA was prepared as described previously (Kroeger & Rowe, 1989). RNA (5 μg) was transferred onto nitrocellulose filter paper using a dot blot apparatus (Bio-Rad Laboratories, Richmond, CA) as described by Maniatis et al. (1982). The filters were preincubated in RNA hybridization buffer (5× SSPE, 1.0% SDS, 50% formamide, and 0.25% nonfat dry milk) at 42 °C. After 4 h, filters were placed in fresh RNA hybridization buffer containing [^{32}P]-labeled probe and incubated an additional 20 h at 42 °C. Filters were then washed as described under Isolation and Analysis of Cellular DNA.

Labeling of DNA Probes. Labeling of DNA by nick-translation was done in the presence of [α-^{32}P]dCTP (3000 Ci/mmol; ICN, Costa Mesa, CA) using a kit obtained from Bethesda Research Labs. The unincorporated nucleotides were separated from the labeled DNA by chromatography through BioGel A-1.5M (Bio-Rad Laboratories). The specific activity of the nick-translated DNA exceeded 1×10^8 cpm/μg. The labeled DNA was denatured by heating for 5 min in a boiling water bath just prior to hybridization.

Oligolabeling primer labeling of DNA was performed as described by Feinberg and Vogelstein (1983) using actin DNA sequences that had been excised and gel-isolated from restriction digests of p5CPS and p57APH plasmid DNAs using the procedure of Maxam and Gilbert (1980). The oligolabeling reactions contained 100 ng of the gel-purified DNA fragment and 5 units of Klenow polymerase in 12.5-μL final volume, and they were incubated at 23 °C for at least 2 h. The labeled DNA was isolated as described above, and the specific activity was greater than 4×10^8 cpm/μg.

Autoradiography. Autoradiography of DNA and RNA filters was done at −70 °C using Kodak XAR-5 film and a DuPont Lightning-Plus intensifying screen.

Quantitation of Radioactivity from RNA and DNA Blots. The radioactivity present in DNA and RNA filter blots was directly quantitated using a Betascope 603 blot analyzer (Betagen Corp., Waltham, MA) as described by the company.

Results

Comparison of Topoisomerase I and II Cleavage Sites on the Drosophila Hsp70 Gene. There are 5–6 copies of the hsp70 gene in *Drosophila* cells located at the 87A and 87C loci of chromosome 3 (Livak et al., 1978; Mirault et al., 1979). These genes are composed of a 0.35-kb conserved regulatory element and a 2.2-kb transcribed coding region as is illustrated in Figure 1. To further analyze the role of topoisomerases I and II in gene expression, we directly compared the binding of these two enzymes to the hsp70 genes in *Drosophila* K_c cells using the inhibitors camptothecin (topoisomerase I specific) and VM-26 (topoisomerase II specific). These drugs interfere with topoisomerase activity by stabilizing a covalent enzyme–DNA intermediate termed the cleavable complex (Liu, 1989). Treatment of this complex with a protein denaturant (i.e., SDS) results in protein-linked DNA strand breaks. These strand breaks conveniently mark the positions of topoisomerase binding sites on DNA and can be readily mapped by Southern analysis using a modification of the indirect end-labeling procedure (Rowe et al., 1986; Kroeger & Rowe, 1989). To

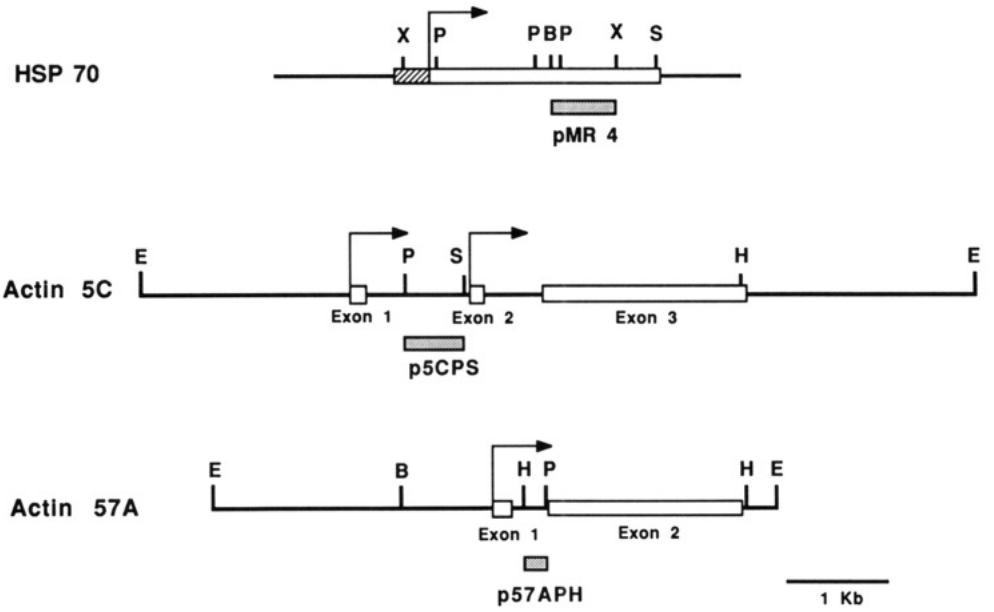

FIGURE 1: Map of *Drosophila* hsp70, actin 5C, and 57A genes. The open boxes represent the transcribed regions present in the mature mRNAs, and the stippled boxes below each schematic designate the subclones used for probes in these studies. The hatched box represents the conserved promoter element of the hsp70 gene. Restriction sites are as designated: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; X, *Xba*I.

specifically map the topoisomerase cleavage sites on the hsp70 gene, DNA isolated from drug-treated cells was restricted with either *Xba*I or *Bam*HI and the DNA resolved on an agarose gel. The DNA was then transferred onto nitrocellulose and hybridized to nick-translated pMR4 probe to identify cleavage sites in the 5′ (*Xba*I digested) or 3′ (*Bam*HI digested) regions of the hsp70 gene (see schematic in Figure 1).

We have previously reported that activation of hsp70 transcription was associated with a dramatic increase in topoisomerase I mediated single- and double-stranded breaks in hsp70 DNA sequences (Kroeger & Rowe, 1989). The double-stranded topoisomerase I breaks were found to result from closely spaced single-stranded breaks that occurred on opposing strands of the hsp70 DNA. It was also found that most of the topoisomerase I cleavage could be abolished by the transcriptional inhibitors Act D and DRB, suggesting that binding of topoisomerase I to the hsp70 gene required ongoing mRNA synthesis. However, this earlier study did not determine whether normal repression of hsp70 mRNA synthesis resulted in similar changes in topoisomerase I binding. To further evaluate the role of topoisomerase I in hsp70 transcription, we monitored topoisomerase I binding during the normal activation and repression of hsp70 transcription. Under normal growth conditions (23 °C), there was no detectable transcription of the hsp70 genes in *Drosophila* K_c cells [see Figure 2, panel C, and also Lindquist (1986)]. However, transcription was quickly activated upon elevating the growth temperature to 37 °C for 10 min. If cells were then allowed to recover at 23 °C for varying lengths of time, there was a gradual down-regulation of hsp70 transcription [panel C and DiDomenico et al. (1982)]. The repression of hsp70 transcription has been shown to closely correlate with the levels of hsp70 protein, suggesting that this gene is subject to autoregulation (DiDomenico et al., 1982). Cleavage of hsp70 DNA sequences by topoisomerase I closely paralleled the level of hsp70 expression (lanes I–N, panels A and B). At 23 °C, there was no detectable cleavage of the hsp70 gene in cells treated with the topoisomerase I inhibitor camptothecin (lane I). Following a 10-min heat shock, cleavage sites appeared throughout the transcribed region of the hsp70 gene with the greatest level of cleavage occurring near the 3′ end of the gene (lane J). During recovery of the cells at 23 °C, there was a uniform loss in topoisomerase I cleavage at all sites within the hsp70 gene (lanes K–N). This result was similar to, but not identical to, what occurs when hsp70 transcription is blocked by the transcriptional inhibitors Act D and DRB (Kroeger & Rowe, 1989). Although inhibition of hsp70 transcription by Act D and DRB results in a loss of topoisomerase I cleavage at most sites in the hsp70 gene, cleavage at the very 5′ and 3′ ends of the gene remains elevated. It has been suggested that this residual cleavage may in part be due to the induction of premature transcripts by Act D and DRB (Kroeger & Rowe, 1989; Chodish et al., 1989).

Quantitation of the radioactivity present in topoisomerase I cleavage bands indicated that there was a close correlation between topoisomerase I cleavage and the level of hsp70 mRNA expression (Figure 2, panel C). These results are consistent with the proposed role of topoisomerase I in the removal of torsional stress in DNA that is generated during transcription and indicate that topoisomerase I cleavage may be a useful marker for regions of DNA that are undergoing active transcription (Kroeger & Rowe, 1989; Stewart et al., 1990).

In contrast to topoisomerase I, topoisomerase II cleavage of hsp70 sequences was observed at 23 °C in the absence of hsp70 expression (lane C, panels A and B). In addition, cleavage was not distributed throughout the gene but instead was localized to the 5′ and, to a lesser extent, the 3′ end of the hsp70 gene which is consistent with results obtained in an earlier study (Rowe et al., 1986). The three major sites at the 5′ end are marked by carets and map to positions +80, +250, and +450 bp relative to the start site of transcription. Heat shocking cells for 10 min resulted in a 2–3-fold increase in topoisomerase II cleavage at the 3′ end of the hsp70 gene (compare lanes C and D in panel B). These induced sites are located at approximately +1900, +2175, and +2625 bp and are designated by carets at the left of the figure. During the 2-h recovery at 23 °C, cleavage at these sites decreased back to control levels (panel B, lanes E–H).

Surprisingly, topoisomerase II cleavage at the 5′ end of the gene, especially at the +80 and +250 bp sites, was lost following the 37 °C heat shock (compare lanes C and D in panel

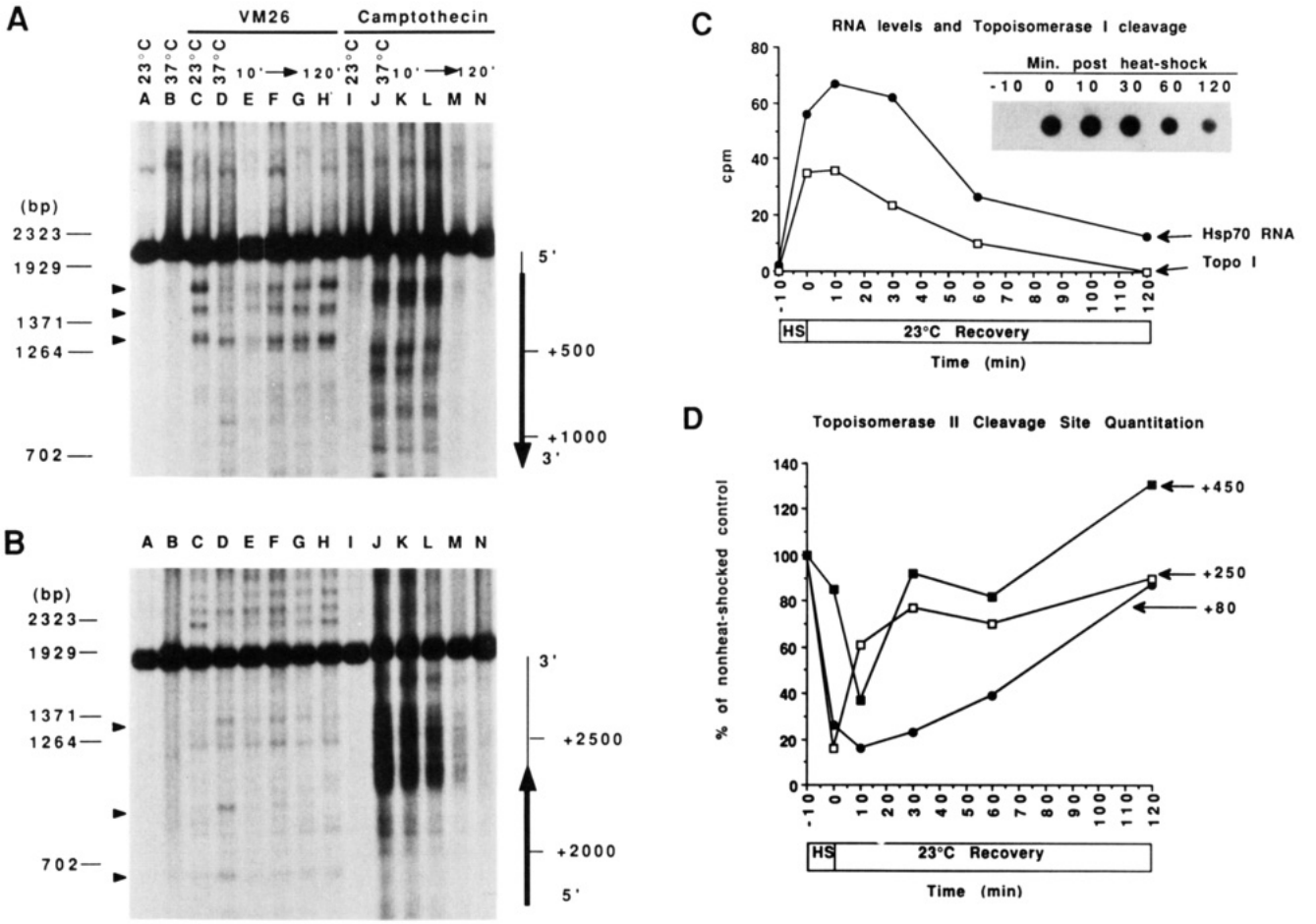

FIGURE 2: Binding of topoisomerases I and II to the hsp70 gene during heat shock and recovery. *Drosophila* K_c cells were heat shocked for 10 min at 37 °C and then allowed to recover for various times at 23 °C. During the last 10 min of the incubation, cells were treated with either VM-26 (20 μM) or camptothecin (20 μM). Cellular DNA was then isolated and analyzed by Southern blotting as described under Materials and Methods. Prior to analysis, the DNA was restricted with either *Xba*I or *Bam*HI to map topoisomerase cleavage sites in the 5′ (panel A) or 3′ (panel B) regions of the hsp70 DNA. Lanes A and B, DNA from cells incubated at 23 and 37 °C, respectively, in the absence of drugs; lane C, DNA from 23 °C cells treated with VM-26; lane D, DNA from cells heat shocked for 10 min at 37 °C and then treated with VM-26 for an additional 10 min at 37 °C; lanes E–H, DNA from cells heat shocked for 10 min at 37 °C and then incubated at 23 °C for 10, 30, 60, and 120 min, respectively, prior to addition of VM-26; lanes I–N, same as lanes C–H except that camptothecin was added. The molecular weight standards (*Bst*EII-digested λ DNA) are noted at the left of each panel, and the region of the hsp70 gene surveyed is schematically depicted at the right. The carets at the left of panels A and B mark topoisomerase II sites that are discussed in the text of the paper. Panel C is a graph comparing the relative levels of hsp70 mRNA and topoisomerase I cleavage during the heat shock recovery experiment. The level of hsp70 mRNA was quantitated from the RNA dot blot (shown in the inset) using a Betascope blot analyzer. The radioactivity present in the major region of topoisomerase I cleavage at the 3′ end of the hsp70 gene (panel B, lanes I–N) was also quantitated using a Betascope blot analyzer. HS refers to the 10-min interval that cells were heat shocked at 37 °C. Panel D is a graph comparing the level of topoisomerase II cleavage at the +80, +250, and +450 bp sites detected in panel A (lanes C–H). The level of cleavage was determined by Betascope blot analysis and plotted relative to the level of cleavage measured in the 23 °C, VM-26-treated sample (lane C, panel A).

A). Cleavage at all three 5′ sites was still depressed even after the cells were allowed to recover for 10 min at 23 °C (lane E, panel A). However, upon further incubation of the cells at 23 °C, there was a gradual recovery of topoisomerase II cleavage at all three sites that correlated with a loss in hsp70 mRNA (lanes E–H, panel A). The relative level of topoisomerase II cleavage at these sites was quantitated, and the data were plotted in panel D. A comparison of panels C and D suggests that there is an inverse relationship between the level of hsp70 mRNA and the binding of topoisomerase II to the 5′ end of the gene. The heat-induced loss in topoisomerase II cleavage at the 5′ end of the hsp70 gene was unexpected since it had previously been reported that heat shock did not appear to affect cleavage at these sites (Rowe et al., 1986). However, the earlier study was different in that VM-26 had been added to the cells just prior to, rather than after, the heat shock. Consistent with these earlier results, we found that addition of VM-26 just prior to heat shock prevented the heat-induced loss of topoisomerase II cleavage at the 5′ end of the hsp70 gene (data not shown). This suggests that once formed, the VM-26-induced enzyme–DNA cleavage complex is not readily reversed, thus explaining why the earlier study did not detect a heat-induced loss in topoisomerase II cleavage at the 5′ end of the hsp70 gene. A schematic summarizing the topoisomerase I and II cleavage sites on the hsp70 gene is presented in Figure 3.

Constant Heat Shock. We also investigated the binding of topoisomerases I and II to the hsp70 gene in cells that were continuously heat shocked at 33 °C (Figure 4). This milder heat shock temperature was used to minimize potential artifacts that might be associated with the higher 37 °C heat shock temperature (i.e., protein denaturation or nonspecific changes in DNA structure). The regulation and kinetics of the heat shock response are also different depending on the temperature at which the heat shock is done, and we were curious whether this would correspondingly alter the time course or pattern of topoisomerase cleavage (Zimarino et al., 1990). Continuous exposure of *Drosophila* K_c cells to a 33 °C heat shock resulted

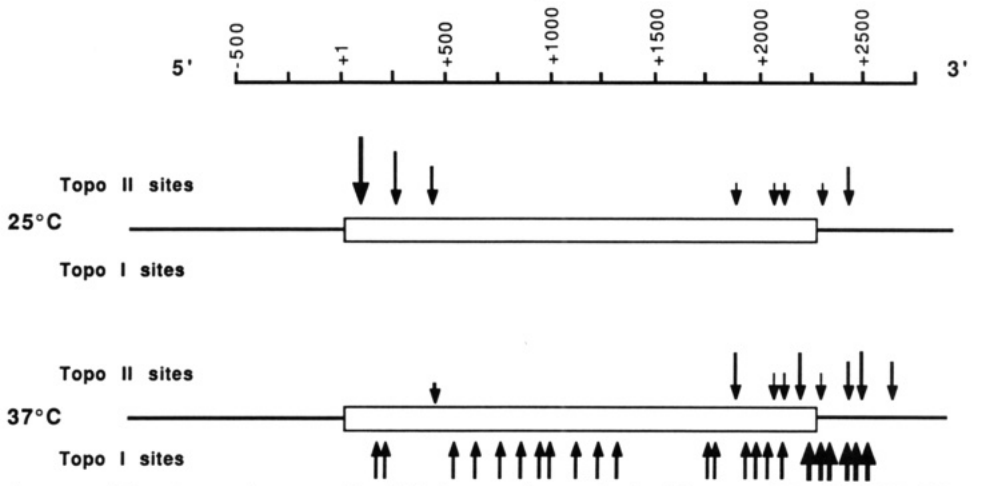

FIGURE 3: Schematic summarizing the topoisomerase I and II cleavage sites on the hsp70 gene at 23 and 37 °C. The open box represents the transcribed region of the hsp70 gene. The arrows above and below the schematics indicate the positions of topoisomerase II and I sites, respectively. The size of the arrows is indicative of the relative strength of the cleavage sites. Above both schematics is a scale that is relative to the start of transcription at nucleotide +1.

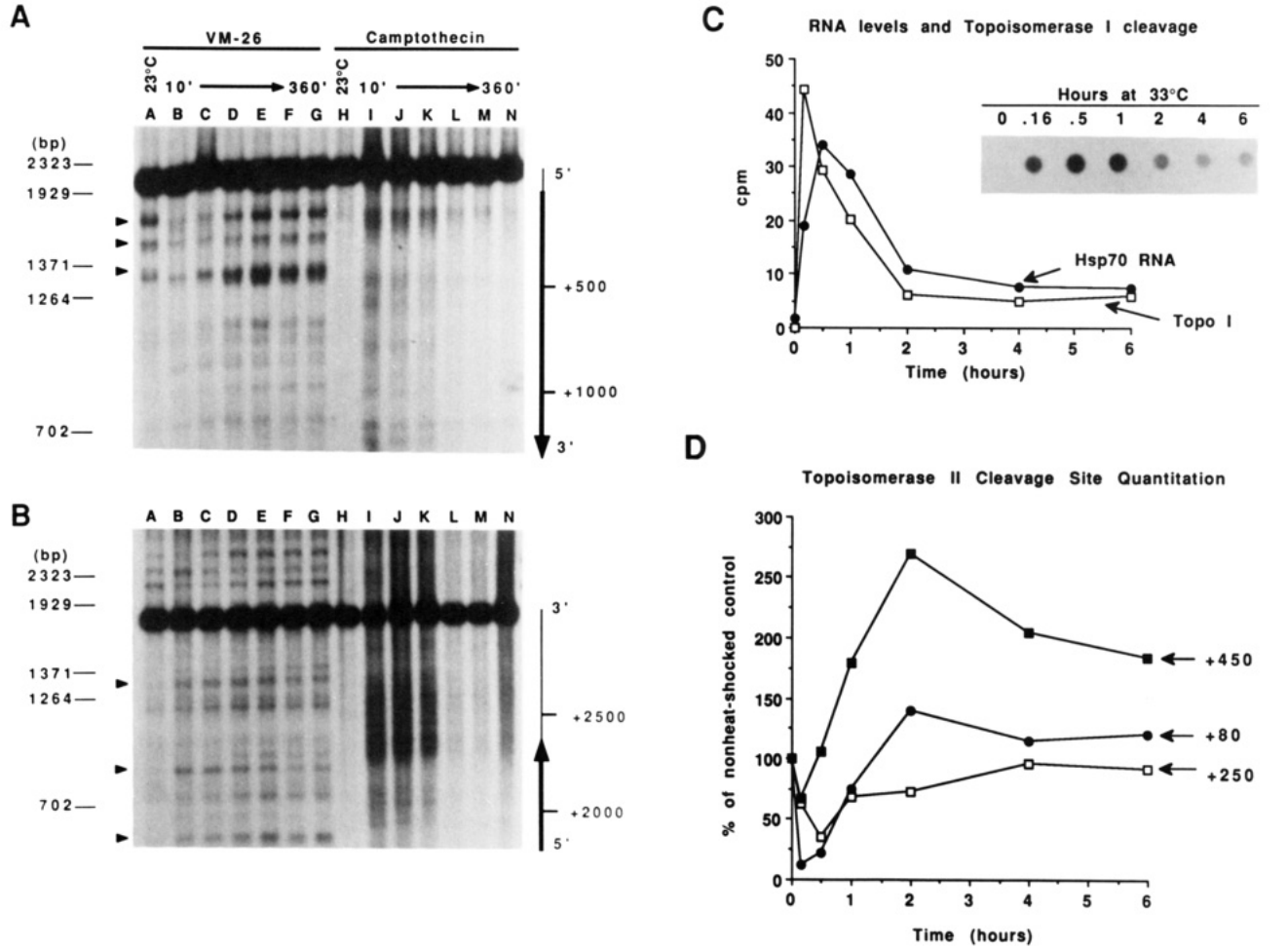

FIGURE 4: Constant heat shock and topoisomerase binding to the hsp70 gene. *Drosophila* K_c cells were incubated at 33 °C for varying lengths of time. During the last 10 min of incubation, cells were treated with 20 μM VM-26 or camptothecin, and topoisomerase cleavage in the hsp70 gene was determined as described in the legend to Figure 2. DNA samples were cut with either *Xba*I or *Bam*HI to map topoisomerase cleavage sites at the 5′ (panel A) or 3′ (panel B) end of the hsp70 gene, respectively. Lane A, DNA from cells incubated at 23 °C in the presence of VM-26; lanes B–G, DNA from cells heat shocked for 10, 30, 60, 120, 240, and 360 min, respectively, at 33 °C prior to the addition of VM-26; lanes H–N are the same as lanes A–G except that camptothecin was added instead of VM-26. Molecular weight standards (*Bst*EII digest of λ DNA) are at the left of each gel, and a schematic representing the region of the hsp70 gene analyzed is depicted at the right. (Panel C) Quantitation of the relative levels of hsp70 mRNA and topoisomerase I cleavage during the 33 °C heat shock was done as described in the legend to Figure 2. (Panel D) Quantitative analysis of the +80, +250, and +450 bp topoisomerase II sites at the 5′ end of the hsp70 gene was done as described in the legend to Figure 2.

in an initial induction of hsp70 mRNA synthesis that peaked between 30 and 60 min (Figure 4, panel C). There was then a rapid decline in the level of hsp70 mRNA resulting from the negative feedback mechanism that down-regulates hsp70 transcription (DiDomenico et al., 1982). Similar to the 37 °C experiment, the level of topoisomerase I cleavage in hsp70

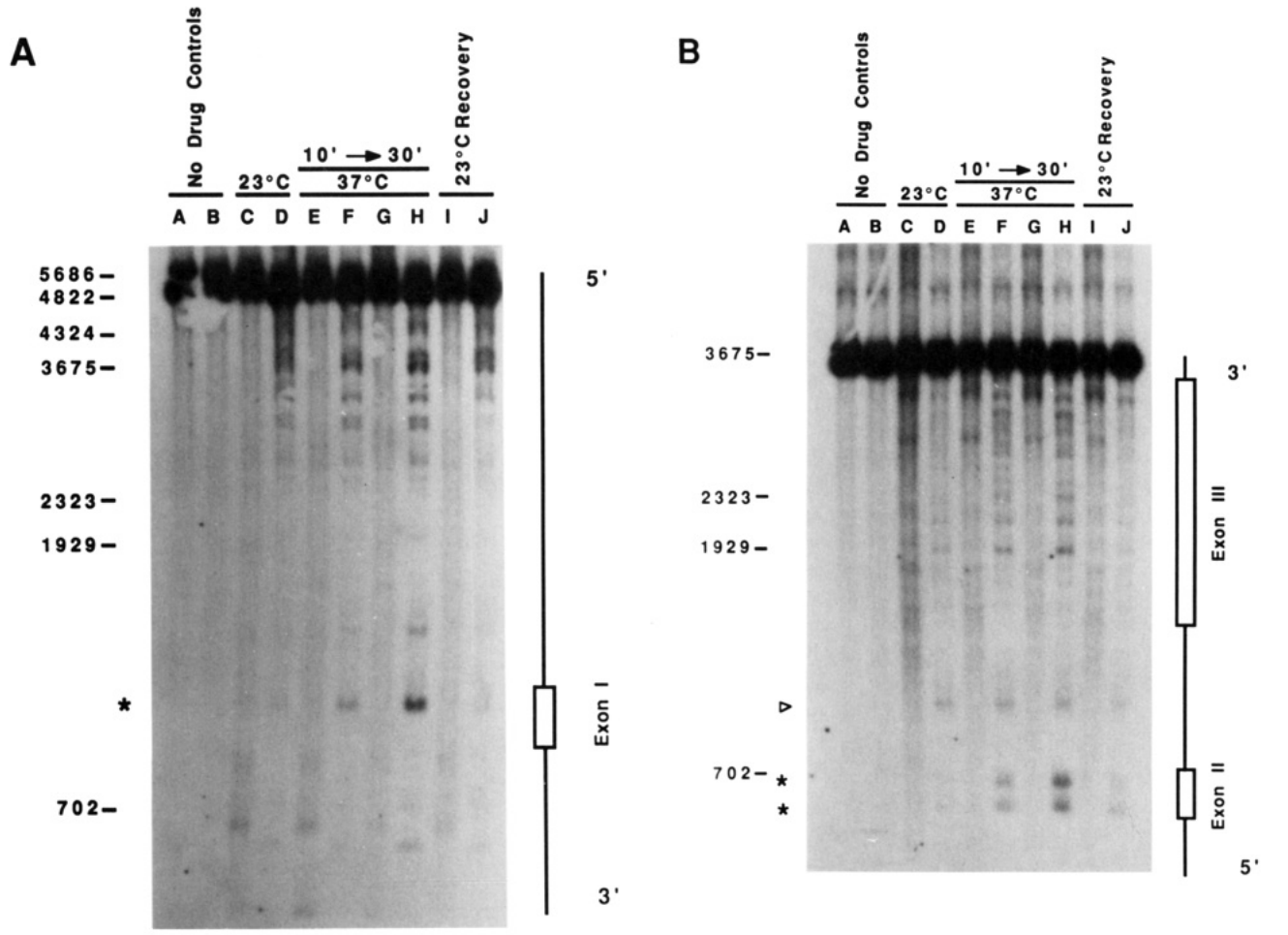

FIGURE 5: Mapping of topoisomerase I and II sites on the actin 5C gene at 23 and 37 °C. *Drosophila* K_c cells incubated at 23 or 37 °C were treated with 20 μM VM-26 (lanes D, F, H, and J) or camptothecin (lanes C, E, G, and I). Total cellular DNA was then isolated and digested with *Sal*I or *Pst*I to analyze for topoisomerase cleavage sites at the 5′ (panel A) or 3′ (panel B) ends of the actin 5C gene as described under Materials and Methods. Lanes A and B, DNA from cells incubated at 23 and 37 °C in the absence of drugs; lanes C and D, DNA from cells incubated at 23 °C for 10 min in the presence of drugs; lanes E and F, DNA from cells heat shocked for 10 min at 37 °C and then treated with drugs for an additional 10 min at 37 °C; lanes G and H, DNA from cells heat shocked for 30 min prior to the addition of drugs for an additional 10 min at 37 °C; lanes I and J, DNA from cells heat shocked for 30 min and allowed to recover for 30 min at 23 °C before the addition of drugs for an additional 10 min at 23 °C. The regions of the actin 5C gene that were examined in each blot are schematically represented at the right side of the figure. Molecular weight standards (*Bst*EII digest of λ DNA) are denoted at the left, and the asterisk and caret mark topoisomerase II cleavage sites that are discussed in the text.

DNA sequences closely correlated with the rise and fall in hsp70 mRNA (panel C). In addition, the pattern of cleavage was identical to that seen when cells were heat shocked at 37 °C (lane I, panels A and B). These results are consistent with a role for this enzyme in removing topological stress generated during hsp70 transcription.

The effects of the 33 °C heat shock on topoisomerase II cleavage were similar to those observed at the 37 °C heat shock. Topoisomerase II cleavage at the 5′ end of the hsp70 gene was initially lost upon incubating the cells at 33 °C (panel A, compare lanes A and B). There was then a time-dependent recovery of topoisomerase II cleavage in this region that coincided with the shut down of hsp70 mRNA expression (panel A, lanes B–G). Quantitation of topoisomerase II cleavage at the +80, +250, and +450 sites (panel D) further emphasizes the correlation between cleavage at the 5′ end of the gene and attenuation of hsp70 expression. In contrast to the 37 °C heat shock results, topoisomerase II cleavage at the +450 site appeared to return more quickly than cleavage at the +80 and +250 sites. Furthermore, after 2 h of heat shock, the level of cleavage at the +450 cleavage site was 2–3-fold greater than that observed prior to the heat shock. Otherwise, the results of the 33 °C heat shock were consistent with those obtained in the 37 °C heat shock and recovery experiment (Figure 2) and suggested that topoisomerase II binding at the 5′ end of the hsp70 gene was related to the repression of hsp70 transcription.

Topoisomerase II cleavage in the 3′ region of the hsp70 gene increased following the 33 °C heat shock (panel B, compare lanes A and B). However, in contrast to the 37 °C heat shock experiment, topoisomerase II cleavage in the 3′ region remained elevated even after 6 h at 33 °C (lane G), a time when hsp70 mRNA levels had subsided.

Actin Gene Expression and Topoisomerase Binding. Although our results suggested that the altered binding of topoisomerases I and II to the hsp70 gene was related to transcription, we could not exclude the possibility that binding was caused by other heat-induced events unrelated to transcriptional activation (i.e., heat-induced changes in DNA structure, growth arrest, protein denaturation, etc.). To address this issue, we analyzed topoisomerase I and II binding to the actin 5C and 57A genes before and after heat shock. The actin 5C gene (see schematic in Figure 1) is constitutively expressed at all stages of *Drosophila* development, and it is regulated through dual promoters that reside at the 5′ end of exons 1 and 2 (Bond-Matthews & Davidson, 1988). Both promoters have been shown to be active in *Drosophila* K_c cells and result in the formation of mRNAs composed of exons 1 and 3 or

exons 2 and 3, respectively. In contrast, the actin 57A gene has a single promoter element adjacent to exon 1. This gene is transcriptionally repressed in *Drosophila* K_c cells and is not activated until later stages of development (Fyrberg et al., 1983). This pair of actin genes represented an interesting combination with which to probe the roles of topoisomerases in transcriptional regulation.

Using an experimental protocol similar to that presented in Figure 2, we compared topoisomerase I and II cleavage sites on the actin 5C gene under different conditions of heat shock and recovery (Figure 5). Topoisomerase sites were mapped in the 5′ or 3′ regions (panels A and B, respectively) of the actin 5C gene by probing *Sal*I or *Pst*I digests of cellular DNA with labeled p5CPS DNA (see Figure 1). The actin 5C gene has been shown to be constitutively expressed in *Drosophila* K_c cells. However, transcription of this gene is arrested when cells are heat shocked at 37 °C (Findlay & Peterson, 1981). As is shown in Figure 5, moderate levels of topoisomerase I cleavage are present throughout the transcribed region of the actin 5C gene at 23 °C, with a majority of the clevage occurring at the 3′ end in exon 3 (panel B, lane C). However, following a 37 °C heat shock, there is a significant reduction in topoisomerase I cleavage (panel B, lanes E and G) that correlates with heat-induced suppression of actin transcription, suggesting that topoisomerase I binding is related to the level of actin mRNA synthesis (Findlay & Peterson, 1981).

Minor topoisomerase II cleavage sites were present throughout the actin 5C gene at 23 °C (panels A and B, lane D). When cells were placed at 37 °C for 10 min, there was a pronounced increase in topoisomerase II cleavage at most sites in the actin 5C gene (lane F). The largest increase in cleavage occurred at three sites in the actin gene. One site was located at the very 5′ end of exon 1 approximately 25 bp downstream from where transcription is initiated from this promoter (panel A, lane F). There were also two sites that appeared in exon 2 approximately 30 and 90 bp downstream from the transcription initiation site of this promoter (panel B, lane F). After 30 min of heat shock, these sites were stimulated greater than 5–7-fold relative to the non heat shocked cells (compare lanes D and H). However, following 30 min of recovery at 23 °C, the level of topoisomerase II cleavage at these sites returned to pre heat shock levels (lane J). There was also one site, present at 23 °C, that remained unchanged throughout the various treatments (Figure 5B, lane D, marked with an open caret at the left). These results are consistent with those that were obtained for the hsp70 heat shock gene and suggest that topoisomerase II binding near the two actin 5C promoters is related to repression of actin transcription.

To further demonstrate that the changes in topoisomerase I and II binding were related to changes in transcription, we examined topoisomerase I and II binding on the actin 57A gene which is inactive at both 23 and 37 °C. Topoisomerase cleavage sites at the 5′ and the 3′ end of this gene were assessed by hybridizing *Pst*I- or *Hin*dIII-digested cellular DNA to the actin 57A subclone p57APH (see schematic in Figure 1). Since topoisomerase I appears to be preferentially associated with transcriptionally active sequences, there should be no binding of this protein to the actin 57A gene at either 23 or 37 °C. Indeed, there was no topoisomerase I cleavage event in the 5′ region of this gene at either temperature (Figure 6, lanes B and E). Topoisomerase I cleavage was also absent in the 3′ region of the gene (data not shown). Correspondingly, heat shock should have no effect on topoisomerase II binding to this gene. At 23 °C, there was one major and two minor

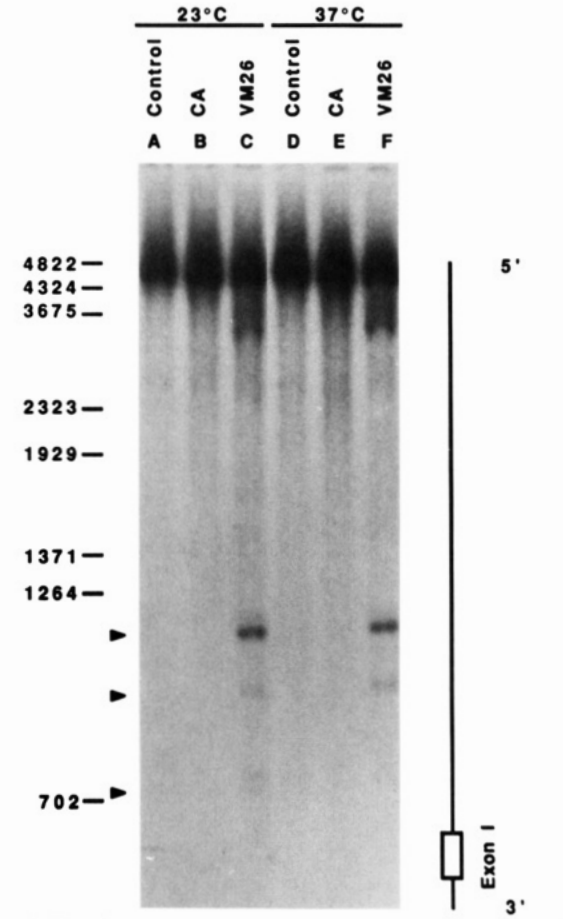

FIGURE 6: Topoisomerase II sites on the actin 57A gene do not change during heat shock. DNA isolated from *Drosophila* K_c cells incubated at 23 °C (lanes A–C) or at 37 °C (lanes D–F) for 30 min was restricted with *Pst*I and analyzed for topoisomerase cleavage in the 5′ region of the actin 57A gene as described under Materials and Methods. Lanes A and D, DNA from cells incubated in the absence of drugs; lanes C and F, DNA from cells treated with 20 μM VM-26 during the last 10 min of the incubation; lanes B and E, DNA from cells treated with 20 μM camptothecin during the last 10 min of incubation. A schematic of the actin 57A gene is shown at the right of the figure. Molecular weight standards (*Bst*EII digest of λ DNA) are denoted at the left, and carets mark topoisomerase II cleavage sites that are discussed in the text.

topoisomerase II sites that could be detected just 5′ to the start site of transcription near exon 1 (Figure 6, lane C). These sites, located at ≈−80, −250, and −370 bp relative to the start site of transcription, were unaffected by the heat shock treatment (lane F). There were no other significant topoisomerase II sites found in exon 2 or in the 3′-flanking regions of this gene at either 23 or 37 °C (data not shown). These results suggest that the changes in topoisomerase I and II binding to the hsp70 and actin 5C genes were related to changes in gene transcription rather than to other unrelated events that were triggered by heat shock. A schematic summarizing the topoisomerase I and II sites on the two actin genes is presented in Figure 7.

Discussion

We have compared the interaction of topoisomerases I and II with several *Drosophila melanogaster* genes in vivo using the inhibitors camptothecin and VM-26. Our experiments suggest that there are significant differences in the interaction of these two enzymes with transcriptionally active and inactive sequences in *Drosophila* DNA. Consistent with our earlier studies, topoisomerase I was found to be preferentially associated with the transcribed regions of transcriptionally active

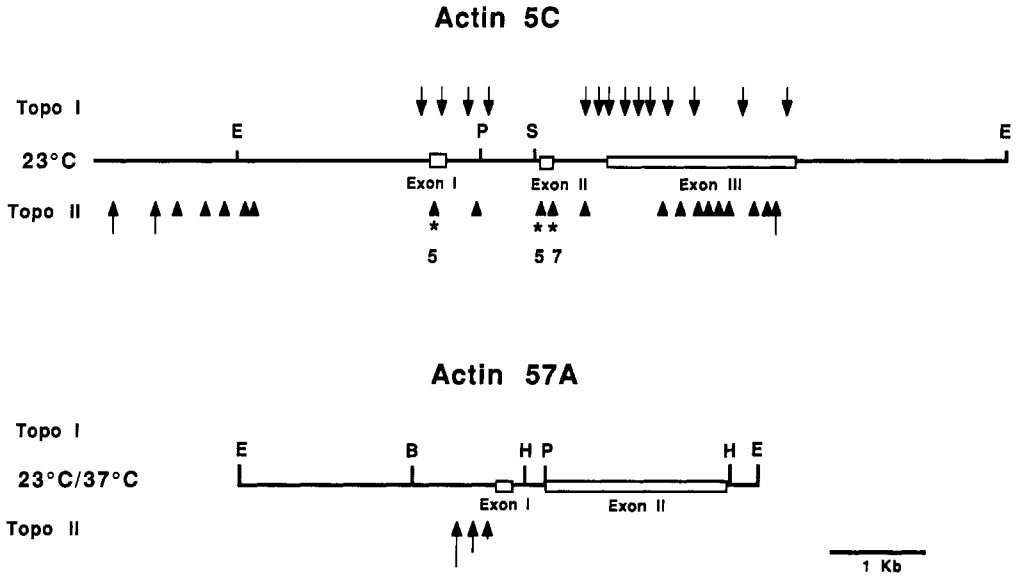

FIGURE 7: Map of topoisomerase I and II sites on the actin 5C and 57A genes at 23 and 37 °C. This schematic summarizes the locations of the topoisomerase I and II sites mapped from the experiments presented in Figures 5 and 6. Topoisomerase I and II sites are depicted with arrows above and below each gene. The asterisk denotes the topoisomerase II cleavage sites that were most significantly enhanced following heat shock at 37 °C, and the numbers below the asterisk indicate the fold enhancement (see text for details). The pattern and level of topoisomerase cleavage on the actin 57A gene were identical at both 23 and 37 °C.

but not inactive hsp70 genes (Kroeger & Rowe, 1989). We have extended the earlier studies by characterizing the association of topoisomerase I with hsp70 sequences during normal repression of this gene. Normal repression of hsp70 mRNA synthesis was accompanied by a uniform loss in topoisomerase I cleavage at all sites within the hsp70 gene. This result is consistent with our earlier study showing that topoisomerase I cleavage could also be abolished by inhibiting hsp70 mRNA synthesis with either Act D or DRB (Kroeger & Rowe, 1989). However, in contrast to normal repression of hsp70 mRNA synthesis, repression by either Act D or DRB did not abolish topoisomerase I cleavage at the very 5′ and 3′ ends of the hsp70 gene. This difference is likely due to the mechanism by which these drugs attenuate transcription. DRB has been shown to inhibit mRNA synthesis by causing premature termination of newly initiated transcripts by RNA polymerase (Chodish et al., 1989). Perhaps the residual topoisomerase I cleavage at the 5′ end of the hsp70 gene is related to the continued synthesis of short transcripts that occurs in the presence of DRB (Kroeger & Rowe, 1989). This, however, cannot account for the residual topoisomerase I cleavage at the 3′ end of the hsp70 gene following inhibition of transcription by either DRB or Act D. We presently have no explanation for this unusual observation.

Recent experiments have shown that the movement of RNA polymerase along a segment of DNA induces positive supercoiling ahead of and negative supercoiling behind the moving transcription complex (Liu & Wang, 1987; Giaever & Wang, 1988; Wu et al., 1988; Tsao et al., 1989). Because topoisomerase I can catalyze the relaxation of both positive and negative supercoils, this enzyme is likely to facilitate transcription by relaxing supercoils which might otherwise accumulate in the DNA and impede the movement of the RNA polymerase complex. In vitro studies have shown that the binding of topoisomerase I to a supercoiled DNA substrate is several orders of magnitude greater than its binding to a relaxed DNA substrate (Camilloni et al., 1988, 1989). This would explain why topoisomerase I is preferentially bound to the transcribed region of active genes, especially near the 3′ ends, where supercoiling would be highest. The role of topoisomerase I in removing supercoils associated with transcription is also supported by studies in yeast mutants lacking topoisomerase I and/or II functions. These studies suggested that topoisomerase I, but not topoisomerase II, was responsible for the relaxation of supercoils generated during transcription of a yeast plasmid DNA [Brill & Sternglanz, 1988; reviewed in Sternglanz (1989)].

In contrast to topoisomerase I, the binding of topoisomerase II to hsp70 and actin genes was not dependent on transcription. Topoisomerase II binding to inactive hsp70 heat shock genes was localized to three sites at the 5′ end that mapped to positions +80, +250, and +450 relative to the start site of transcription. Following heat-induced activation of hsp70 transcription, these sites were lost with a concomitant increase in topoisomerase II binding at the 3′ end of this gene. Recovery of the cells from the heat shock resulted in a return of topoisomerase II binding at the 5′ end of the hsp70 gene that was temporally coupled to the repression of hsp70 mRNA synthesis. Similar results were also obtained when hsp70 transcription was induced by milder heat treatments or with $CdCl_2$ (data not shown), suggesting that the changes in topoisomerase II binding were related to transcription rather than to other effects caused by high temperatures. The preferential binding of topoisomerase II at the 5′ end of inactive hsp70 genes suggested that this enzyme might function in the repression of hsp70 transcription. This role is also suggested by the preferential association of topoisomerase II with the 5′ ends of other repressed genes. Repression of actin 5C transcription by heat resulted in a 5–7-fold increase in topoisomerase II binding near the start sites of transcription in exons I and II. Binding at these sites was reversed following recovery of the cells from the heat shock. Topoisomerase II was also present at the 5′ end of the transcriptionally inactive actin 57A gene. Binding to this gene was not altered by heat shock, suggesting that heat per se was not responsible for the changes in topoisomerase II binding to the 5′ ends of the hsp70 and actin 5C genes. Instead, these results suggest that binding may be related to events involved in the repression of gene transcription.

The preferential association of topoisomerase II with the 5′ ends of transcriptionally repressed hsp70 and actin genes is intriguing and may reflect structural differences in the

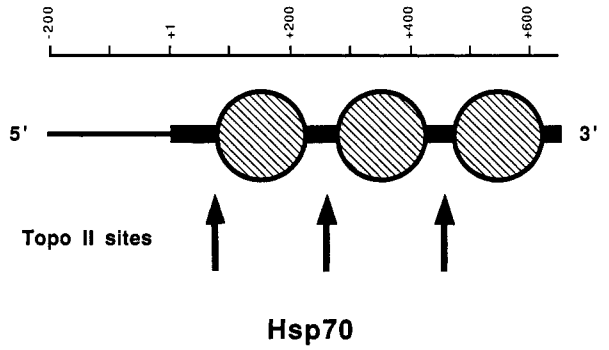

FIGURE 8: Localization of topoisomerase II cleavage to internucleosomal sites. The schematic illustrates the relative positions of topoisomerase II cleavage sites (arrows) and nucleosomes (hatched circles) at the 5′ region of the hsp70 gene. The nucleosome pattern was determined by several laboratories from nuclease digestion studies (Levy & Noll, 1980; Udvardy & Schedl, 1984).

chromatin between active and inactive genes. We have compared the in vivo topoisomerase II binding sites at the 5′ end of the hsp70 gene with the nucleosomal organization previously reported for this region (Levy & Noll, 1980; Udvardy & Schedl, 1984). As illustrated in Figure 8, the topoisomerase II cleavage sites at +80, +250, and +450 fall within internucleosomal spacer regions. A similar finding has also been made for the chicken globin genes (Reitman & Felsenfeld, 1990). In addition, in vitro studies with nucleosome-reconstituted SV40 DNA have shown that topoisomerase II cleavage sites are confined to internucleosomal regions, indicating that nucleosomal positioning was an important determinant affecting topoisomerase binding to DNA (Capranico et al., 1990). The localization of topoisomerase II to internucleosomal regions at the 5′ and/or 3′ ends of genes in vivo may simply reflect the accessibility of these regions of chromatin to enzyme binding. However, it is also possible that topoisomerase II binding at these sites may play a direct role in regulating the chromatin structure in these regions. The loss in topoisomerase II binding at the 5′ end of the hsp70 gene following heat shock may be due to displacement of this enzyme by RNA polymerase or other proteins involved in transcription of this gene. Disruption of the duplex character of DNA during transcription may also contribute to the loss in topoisomerase II binding at the 5′ end of the hsp70 gene. This later interpretation is supported by preliminary experiments in our lab showing that purified *Drosophila* topoisomerase II preferentially cleaves double-stranded versus single-stranded DNA templates (Kroeger & Rowe, data not shown).

Recent studies have demonstrated that RNA polymerase II is transcriptionally engaged at the 5′ end of the uninduced *Drosophila* hsp70 and hsp26 genes (Rougvie & Lis, 1988, 1989, 1990). Apparently, RNA polymerase is able to initiate transcription but is arrested after forming a small transcript approximately 25 nucleotides in length. Premature termination of transcription has also been shown to occur in a variety of other eukaryotic genes transcribed by RNA polymerase II [reviewed in Proudfoot (1989)]. The mechanism underlying premature termination of hsp70 mRNA synthesis is unknown, but it has been suggested that movement of the polymerase complex may be blocked by the presence of binding factors or nucleosomes positioned at the 5′ end of the gene (Rougvie & Lis, 1989). The three topoisomerase II binding sites at the 5′ end of the hsp70 gene reside just downstream of where hsp70 transcription is prematurely arrested. Deletion mutant studies have also suggested that this region contains an element that negatively regulates hsp70 transcription (Corces & Pellicer, 1984). Whether or not topoisomerase II is directly involved in repressing hsp70 transcription is unclear. Interestingly, treatment of cells with the topoisomerase II inhibitor VM-26 induces hsp70 transcription approximately 7-fold, suggesting that this enzyme may have a function in the negative regulation of hsp70 transcription (Rowe et al., 1986). Possibly, this enzyme facilitates changes in the topological structure of chromatin that are ncessary for repression of hsp70 transcription. This might also explain the preferential association of topoisomerase II with the 5′ ends of inactive actin 5C and 57A genes. Alternatively, the binding of topoisomerase II at the 5′ end of the inactive hsp70 gene may reflect a role of this enzyme in relaxing DNA supercoils generated during the synthesis of premature hsp70 transcripts. To further evaluate the role of topoisomerase II in transcription, we plan to investigate whether deletion or addition of topoisomerase II binding sites at the 5′ end of the hsp70 gene alters the regulation of hsp70 transcription.

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