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Localization of Specific Topoisomerase I Interactions within the Transcribed Region of Active Heat Shock Genes by Using the Inhibitor Camptothecin

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Camptothecin stabilizes the topoisomerase I-DNA covalent intermediate that forms during the relaxation of torsionally strained DNA. By mapping the position of the resultant DNA nicks, we analyzed the distribution of the covalent intermediates formed on heat shock genes in cultured *Drosophila melanogaster* cells. Topoisomerase I was found to interact with the transcriptionally active genes *hsp22*, *hsp23*, *hsp26*, and *hsp28* after heat shock but not with the inactive genes before heat shock. The interaction occurred predominantly within the transcribed region, with specific sites occurring on both the transcribed and nontranscribed strands of the DNA. Little interaction was seen with nontranscribed flanking sequences. Camptothecin only partially inhibited transcription of the *hsp28* gene during heat shock, causing a reduced level of transcripts which were nonetheless full length. Topoisomerase I also interacted with the DNA throughout the transcriptionally active *hsp83* gene, including an intron, in both heat-shocked and non-heat-shocked cells. The results point to a dynamic set of interactions at the active locus.

In procaryotes, it is clear that the degree of DNA superhelicity can profoundly influence the utilization of specific promoters (reviewed in reference 16). Similarly, superhelicity of DNA in eucaryotes may be an important parameter of chromatin structure related to the potential for transcription (32, 45). When DNA is introduced into frog oocytes, circular templates are transcribed at 500 times the level of linear templates (24). Preference for the circular molecules suggests that a topologically constrained structure may be necessary to generate or utilize a suitable template for efficient transcription. Ryoji and Worcel (39) have described the assembly of so-called "dynamic" and "static" chromatin structures in the *Xenopus* oocyte system. The dynamic structure, which is reported to be that which is transcriptionally active, appears to be torsionally strained, while the transcriptionally inactive static structure is not torsionally strained.

It has long been recognized that the advancement of the transcription complex along the DNA would be facilitated by a swivel point which would allow the release of superhelicity generated by the passage of RNA polymerase along the DNA (48). The problem of superhelicity might be particularly severe if components of the transcription process are restrained by attachment to a nuclear matrix (28, 37). A role for a swivel activity in the transient unfolding of the chromatin fiber might also be postulated. A change in the compaction of the chromatin fiber as well as a perturbation of the histone-DNA interactions has been shown to occur at active genes (50, 52). The perturbation of the nucleosome array appears to correlate with the level of transcription; among the heat shock genes of *Drosophila melanogaster*, those transcribed at higher levels are more sensitive to cleavage by DNase I and methidiumpropyl-ferrous EDTA than are those transcribed at lower levels (6).

Two classes of enzymes are thought to play important roles in modulating DNA topology (for reviews, see references 14, 46, and 49). Topoisomerases I and II alter DNA topology by introducing transient single- and double-strand breaks, respectively, in the DNA. The breaks are resealed by the enzymes themselves after relaxation of the DNA. The topoisomerase II of some procaryotes can also function as an ATP-dependent gyrase, causing an increase in DNA superhelicity. While a gyrase activity has been inferred in eucaryotic systems (39, 45), to date such an activity has not been isolated.

Both topoisomerases appear to interact with DNA in vivo in the vicinity of genes. By using both immunological and biochemical techniques, topoisomerase II has been found to be identical to protein I of the metaphase chromosomal scaffold of mammalian cells (13). As such, topoisomerase II could play a direct role in organizing topological domains. Studies of topoisomerase II in yeasts have shown that it plays an essential role in resolving DNA replication intermediates (12, 26). By using the drug VM26, Rowe et al. (38) have shown that topoisomerase II can interact in vivo near the 5′ and 3′ ends of the *hsp70* heat shock gene. Immunofluorescence studies of interphase nuclei and polytene chromosomes of *D. melanogaster* show a broad distribution of topoisomerase II within the nuclei and chromosomes, respectively (3, 25). In contrast, immunofluorescense studies of *Drosophila* polytene chromosomes have shown that topoisomerase I is restricted to a subset of loci, apparently being recruited to those that are transcriptionally active (15, 43). By using protein-DNA cross-linking, topoisomerase I has been shown to be associated with the transcribed region and not with the nontranscribed flanking regions of active heat shock genes (19). Topoisomerase I also interacts with the histone and copia genes (19).

Here we present a more precise analysis of the topoisomerase I interaction with DNA in vivo by using a specific inhibitor of topoisomerase I, camptothecin. Camptothecin inhibits topoisomerase I activity by stabilizing the covalent protein-DNA intermediate (27). When the reaction complex is treated with sodium dodecyl sulfate, (SDS), topoisomerase I is recovered covalently coupled to the DNA. The nick located where the topoisomerase I cut the

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DNA remains when the protein moiety is removed with proteinase K; the position of the nick can be mapped with DNA blots (42). We have focused our analysis on several heat shock genes in *D. melanogaster*, as the transcriptional state of these genes is easily manipulated. Very low levels of topoisomerase I activity are detected in association with the inactive genes in noninduced cells, but high levels are detected in association with the highly transcribed genes in heat-shocked cells. The topoisomerase I is distributed in a nonrandom fashion within the gene, interacting with both the transcribed and nontranscribed strands of the DNA.

MATERIALS AND METHODS

Camptothecin was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Institutes of Health, Bethesda, Md., and dissolved as a 10 mM stock in dimethyl sulfoxide. This stock was thawed and frozen numerous times over a period of many months with no apparent change in activity. All other reagents were of typical laboratory grade.

Preparation of DNA from camptothecin-treated cells. Schneider S2 cells were grown in tissue culture flasks to densities of 5×10^6 to 1×10^7 cells per ml. Flasks of cells were heat shocked by gently shaking the flask in a 36°C water bath for 20 to 30 min. Camptothecin was added to the cells, and they were maintained at their respective temperatures for the indicated lengths of time. After their collection, usually with a 30-s spin at room temperature in a microcentrifuge, the cells were suspended in 150 mM NaCl–10 mM EDTA–10 mM Tris chloride (pH 8.0). One-fifth volume of 5% SDS–0.1 M EDTA (pH 7.5) was added to lyse the cells. DNA was prepared from the lysate with proteinase K treatment (10 to 50 μg/ml for at least several hours at 37°C), phenol-chloroform extraction, and ethanol precipitation. Restriction digests were performed by the instructions of the manufacturer. Both alkaline and neutral agarose gel electrophoresis were carried out as described by Maniatis et al. (33). Alkaline gels were stained with ethidium bromide in Tris-acetate-EDTA electrophoresis buffer (33) and destained in water. Neutral gels were stained in water. DNA to be blotted from the agarose gels was depurinated, denatured, and neutralized essentially as described by Wahl et al. (47), with modifications described by Gilmour and Lis (18). DNA was blotted to nitrocellulose essentially as described by Southern (42). Hybridization conditions were in the presence of dextran sulfate but no formamide and at 65°C for overnight (47).

RNA analysis. Cells were collected and suspended in 150 mM NaCl–10 mM EDTA–50 mM Tris chloride (pH 8.0) and lysed with an equal volume of 1% SDS–25 mM EDTA–50 mM NaCl–10 mM Tris chloride (pH 8.0) (23). Lysates were phenol extracted (equilibrated to pH 8.0 with 1 M Tris chloride), ether extracted and ethanol precipitated. The dried precipitate was processed and analyzed as described by Steiner et al. (43).

Mobility shift assay. DNA for the mobility shift assay was prepared essentially as described by Gocke et al. (20). After chloroform extraction and ethanol precipitation, the DNA was cut with a restriction enzyme in the presence of 0.5 mM phenylmethylsulfonyl fluoride. After digestion, the samples were ethanol precipitated and dissolved in 0.5% SDS–50 mM Tris chloride (pH 8.0)–10 mM EDTA. Half of each sample was treated with proteinase K and ethanol precipitated, while the remainder was simply ethanol precipitated. Samples were run on alkaline agarose gels as described by Maniatis et al. (33). The gels were then stained with ethidium bromide in Tris-acetate-EDTA, photographed, and subsequently blotted as described above.

Subclones used for the generation of hybridization probes. Probe 1 is a *Bam*HI-*Hin*dIII fragment from 88.1 (9). M13 vectors are described by Messing (34). Probes 2a and 2b are subclones of a *Hin*dIII-*Cla*I fragment from the clone 26.3 inserted, respectively, into *Hin*dIII-*Acc*I-cut M13mp8 and M13mp9. Clone 26.3 is composed of the *Bam*HI-*Sal*I fragment from aDm202.7 (10) inserted into *Bam*HI-*Sal*I-cut pUC13. The *Hin*dIII site in 26.3 is derived from the polylinker of pUC13 and lies very close to the *Sal*I site. Probes 3a and 3b are subclones of the *Hin*dIII-*Eco*RI fragment from the clone 88.1 (9) inserted, respectively, into *Hin*dIII-*Eco*RI-cut M13mp8 and M13mp9. Probes 4a and 4b are subclones of the *Bam*HI-*Eco*RI fragment from cDm 4.46 (36) inserted, respectively, into M13mp8 and M13mp9.

Preparation of strand-specific probes. Strand-specific probes were synthesized by the Klenow fragment of DNA polymerase by using a M13 pentadecamer sequencing primer (403-2; BioLabs) and radiolabeled nucleotides (usually dCTP and dATP at 6,000 Ci/mmol). After a 1- to 3-h incubation at room temperature, DNA was ethanol precipitated by adding one-half volume of 7.5 M ammonium acetate, 2 volumes of ethanol, and 400 ng of sonicated salmon DNA. The ethanol precipitate was rinsed once with cold 75% ethanol and dried. The DNA was dissolved in 50 mM NaOH and run on a 1% Tris-borate-EDTA agarose gel (33) whose sample wells were previously soaked for about 5 min with 50 mM NaOH. The newly synthesized DNA was detected in the wet gel by autoradiography, excised (approximately 0.5 ml in volume) and boiled in about 1 to 2 ml of water with 500 μg of salmon sperm DNA. This was diluted to 15 to 30 ml with hybridization mix and used to detect specific strands on the DNA blots.

RESULTS

Camptothecin induces extensive cutting in a region containing actively transcribed heat shock genes. We first assessed whether camptothecin induces cleavage of the region containing the small heat shock genes *hsp26*, *hsp23*, and *hsp28* in cultured *Drosophila* cells (Fig. 1). These genes from locus 67B have been previously cloned and mapped (9, 11, 41). A special effort was made to reduce the dissociation of the topoisomerase I-DNA complexes during the early stages of the DNA purification procedure. Cells were collected rapidly, briefly suspended in buffer, and lysed with the addition of SDS. The lysates were treated with proteinase K, and the DNA was purified by phenol extraction, then cut with restriction enzymes, and finally analyzed by DNA blotting and hybridization. The positions of the camptothecin-induced cuts were assessed by using an indirect end-labeling technique (35, 51). Genomic DNA bound to the nitrocellulose was probed with radioactive DNA homologous to a short region abutting a restriction enzyme cut in the genomic DNA. One large "parental" band was detected due to cutting by the restriction enzyme at each end. Additional smaller bands can be ascribed to cuts in the DNA introduced in vivo as a result of camptothecin treatment. The sizes of the bands reflect the distance of these cuts from the restriction site abutting the probe.

Figure 2 shows that a *Bam*HI fragment containing three closely spaced heat shock genes was extensively cut in heat-shocked, camptothecin-treated cells. The cuts were located most prominently in the actively transcribed regions.

In contrast, this region was not extensively cut in noninduced cells nor in cells that were induced but not treated with camptothecin. The very weak cuts that were detected in noninduced cells occurred at positions similar to those in heat-shocked cells, suggesting that they may originate from cells that were inadvertantly stressed. The DNA cutting in heat-shocked cells was specific; we detected no cutting of the histone genes (examined by hybridization of a similar blot with an appropriate probe) or of prominent restriction fragments detected on the ethidium bromide-stained agarose gels (data not shown). The absence of detactable cutting in the histone genes probably stems from their overall low level of transcription in these nonsynchronously growing cells (1).

The DNA was examined by using both neutral and alkaline gels, although we anticipated that only single-stranded nicks would be introduced into the DNA. Surprisingly, subfragments were detected on the neutral gels, indicating that camptothecin also induces double-stranded breaks. However, the intensity of the parental band on the alkaline gel appeared weaker than on the neutral gel, suggesting that there were more single- than double-strand breaks. Moreover, essentially all DNA molecules were nicked since so little of the parental band remains after camptothecin treatment. We observed that the single- and double-stranded cuts accompany one another over a wide range of drug treatments, the lowest tested being 10 nM camptothecin for a 5-min period (data not shown).

Camptothecin-induced cuts occur within the transcribed regions of the small heat genes *hsp28* and *hsp23*. The positions of the camptothecin-induced cuts on individual strands of DNA were assessed by using strand-specific probes prepared from inserts in M13 phage DNA. Genomic DNA purified from the treated cells was cut with a restriction enzyme and run on alkaline gels to separate individual strands of the DNA. Figure 3, lanes 4, shows several camptothecin-induced cuts within *hsp28* and *hsp23* in heat-shocked cells. In this case, the genomic DNA was cut with *Eco*RI, and the fragments were analyzed by using probes 3a and 3b (Fig. 1). Much weaker cuts were observed in the untreated sample (Fig. 3, lane 1); their origins are unknown. Each strand displayed its own unique pattern of camptothecin-induced cuts. These cuts were localized within the transcribed regions of the genes *hsp28* and *hsp23*. Some cuts, most notably the strong cut near the 3′ end of the *hsp28*, occur in both strands. These coincident cleavage events probably account for the double-stranded cutting observed on neutral gels. Somewhat surprisingly, there are three relatively prominent cleavage sites on the transcribed strand, whereas there is only one prominent cleavage site on the nontranscribed strand.

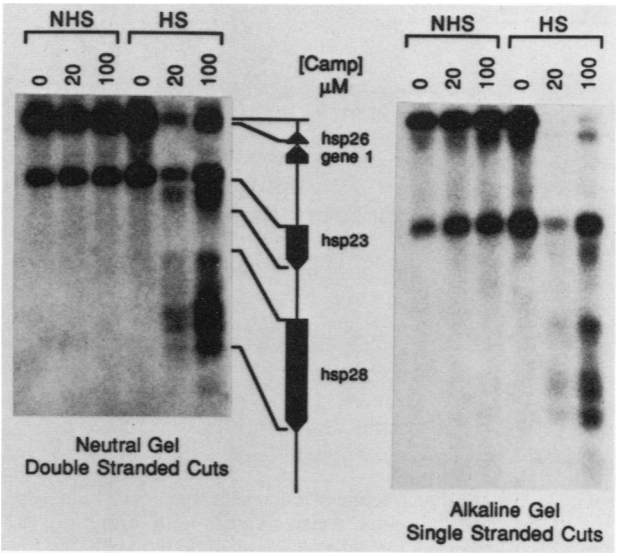

FIG. 2. Camptothecin induces both single- and double-strand cuts in the vicinity of the small heat shock genes in heat-shocked but not in noninduced cells. The region containing the small heat shock genes was analyzed in genomic DNA from cells that were either heat shocked (HS) or not (NHS) and were treated with 0, 20, or 100 μM camptothecin for 20 min. Each DNA sample was cut with *Bam*HI and a portion was run on either a 1% neutral or a 1% alkaline agarose gel. After transfer of the DNA to nitrocellulose, the region containing the small heat shock genes was detected with probe 1 shown in Fig. 1. *Bam*HI digestion produces a major band of 12 kilobases corresponding to the intact genomic DNA fragment. Camptothecin induces extensive double- and single-strand cuts which result in a complex pattern of lower-molecular-weight fragments. The origin of the band below the parental band and present in all lanes is unknown. The ethidium bromide-stained gel showed that less DNA was loaded in the HS, 20 μM camptothecin lane than in other lanes.

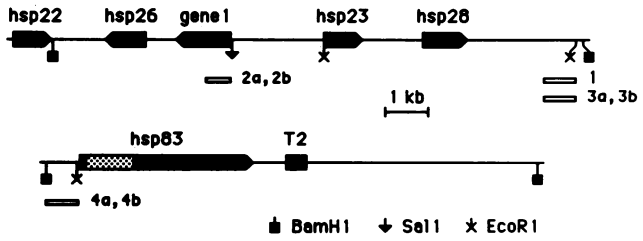

FIG. 1. Maps and relevant probes for the regions containing the small heat shock genes and *hsp83*. The map containing gene *1* and the small heat shock genes, *hsp22*, *hsp26*, *hsp23*, and *hsp28* is derived from that of Sirotkin and Davidson (41). The map containing *hsp83* and *T2* is derived from O'Connor and Lis (36) and Hackett and Lis (22). The black arrows delineate transcribed regions with the arrow heads pointing in the direction of transcription. The *hsp83* intron is delineated by the stipled region. The orientation of the *T2* transcript is not known. Only relevant restriction sites are shown. Various probes and their locations relative to the maps are depicted by the white rectangles situated below each map. Probe 1 is produced by nick translation of the *Bam*HI-*Hin*dIII restriction fragment from the clone 88.1 (9). Probes 2a, 2b, 3a, 3b, 4a, and 4b are produced by primer extension by the Klenow fragment of DNA polymerase by using single-stranded M13 phage DNA as the template. Hence, each of these probes is homologous to only one DNA strand.

Camptothecin induces the covalent attachment of a protein to the 3′ end of DNA strands. The induction of double-stranded cuts by camptothecin is more suggestive of a topoisomerase II activity than a topoisomerase I activity. Therefore, we characterized the product of the camptothecin-induced cut in more detail. Gocke et al. (20) have shown that the topoisomerase I-DNA adducts remain in the aqueous phase after chloroform extraction of a nuclear lysate. The DNA is then suitable for digestion with restriction enzymes. It is known that topoisomerase I forms a covalent linkage with the 3′ end of the nicked DNA, while topoisomerase II forms a covalent linkage with the 5′ end of the cleaved DNA (49). To determine to which end of the DNA the camptothecin-induced protein complex was attached, we examined the mobility on alkaline agarose gels of specific single-stranded DNA fragments before and after protease treatment. The presence of protein will decrease

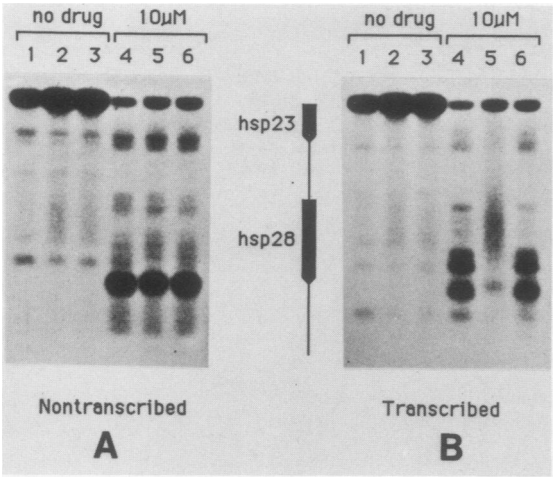

FIG. 3. Camptothecin-induced cuts near the *hsp28* and *hsp23* genes. Heat-shocked cells were treated with either 10 μM camptothecin or an equivalent volume of dimethyl sulfoxide for 3 min. at 36°C and then collected by a 5-min spin in a clinical centrifuge. Cells were lysed in 1% SDS, and a portion of the lysate was treated immediately with proteinase K, phenol-chloroform extracted, and ethanol precipitated. The remaining DNA was not treated with protease but extracted several times with chloroform followed by ethanol precipitation. The DNA was cut with *Eco*RI in the presence of 0.5 mM phenylmethylsulfonyl fluoride and then ethanol precipitated. Precipitates were dissolved in 0.5% SDS–50 mM Tris (pH 8.0)–10 mM EDTA, and half was treated with proteinase K. Both samples were again ethanol precipitated and then run on an alkaline gel, along with the sample treated immediately with proteinase K. Cuts on specific strands were detected with strand-specific probes 3a and 3b (Fig. 1). (A) Cuts in the nontranscribed strand. Lanes: 1, 2, and 3, cells not treated with camptothecin; 4, 5, and 6, cells treated with camptothecin. Lanes 1 and 4 are samples that were treated with proteinase K before the organic extraction. Lanes 2 and 5 are samples that were never treated with proteinase K. Lanes 3 and 6 are samples that were treated with proteinase K after restriction digestion, just before electrophoresis. The probe detects cleavage fragments whose 5′ ends were generated with camptothecin. (B) The same blot as in panel A hybridized with a probe specific for the transcribed strand. This probe detects cleavage fragments whose 3′ ends were generated with camptothecin.

the mobility of a fragment, while protease treatment should return the mobility to that of naked DNA. Figure 3B, lane 5, shows that fragments whose 3′ ends were generated by camptothecin treatment were retarded before but not after protease treatment. The smeared appearance of the DNA in Fig. 3B, lane 5, is probably due to the bound topoisomerase I. This protein is generally very sensitive to partial proteolysis (15, 29). In contrast, Fig. 3A, lane 5, shows that fragments whose 5′ ends were generated by camptothecin were not retarded even in the absence of protease treatment. A similar analysis of this filter with a strand-specific probe homologous to the region abutting the *Eco*RI site in *hsp23* (Fig. 1) shows that the strong cuts detected in Fig. 3A do have protein covalently linked to their 3′ ends (data not shown). These observations preclude the possibility that these cuts were due to topoisomerase II, which would result in the covalent attachment of the protein to the 5′ end of the DNA. Moreover, it shows that the smeared appearance of the DNA in Fig. 3B, lane 5, is specific for those detected fragments and not a general problem with that DNA sample. Protein does not appear to be associated with either the 5′ or 3′ ends of the DNA at the weak cuts in the untreated samples since no shift in the mobility of the DNA fragments occurs in the absence of protease treatment (compare Fig. 3A and B, lanes 2 and 3).

Additional experiments support the conclusion that topoisomerase II is not responsible for the observed cutting. The drug VM26 traps topoisomerase II on DNA as a protein-DNA complex similar to the camptothecin-induced complex of topoisomerase I with DNA (8, 38). Side-by-side comparison of the cutting patterns induced by VM26 and by camptothecin treatment of cells revealed that the patterns are very different, suggesting that the two drugs have different targets (S. Suryaraman and D. Gilmour, unpublished data). Moreover, 100 μg of novobiocin per ml, an inhibitor of topoisomerase II, did not inhibit camptothecin-induced strand cleavage (data not shown). In contrast, Rowe et al. (38) have shown that novobiocin does inhibit VM26-induced DNA cleavage.

Camptothecin only partially inhibits transcription of *hsp28*. Topoisomerase I interacts primarily with the DNA of the transcribed regions of the active heat shock genes (Fig. 3). It might be anticipated that camptothecin would inhibit transcription, especially if the drug considerably lengthens the lifetime of the protein-DNA intermediate. Figure 4, lanes 4 and 5, shows that camptothecin only partially inhibits transcription of *hsp28* in heat-shocked cells. The effect is seen regardless of whether the drug is administered just before or just after heat shock. Similar levels of transcriptional inhibition were also seen with 0.1 and 1 μM camptothecin (data not shown). The transcripts produced appear to be of normal size (even on longer autoradiographic exposures), suggesting either that most of the transcripts are completed or the prematurely terminated transcripts are rapidly degraded. Figure 4, lane 2, shows that camptothecin does not by itself induce transcription of this heat shock gene.

Camptothecin induces frequent cleavage in *hsp26* and *hsp22* but not in gene *1*. Three additional genes reside upstream of *hsp23*. Two of these, *hsp26* and *hsp22*, are heat shock-inducible genes, while the third, gene *1*, is relatively inactive in these cells. Figure 5, lanes 4, shows that topoisomerase I was predominately associated with the actively transcribed heat shock genes and not with gene *1*. As in the case of *hsp28* (Fig. 3), topoisomerase I was not detected on the nontranscribed sequences immediately flanking the *hsp26*

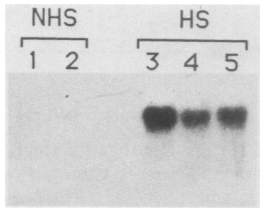

FIG. 4. Camptothecin partially inhibits transcription of *hsp28*. The level of *hsp28* RNA was measured from an RNA blot. Cells were treated in the following ways. Lanes 1, RNA from noninduced cells treated with 0.2% dimethyl sulfoxide; 2, RNA from noninduced cells treated with 10 μM camptothecin for 40 min; 3, RNA from cells heat shocked for 40 min; 4, RNA from cells treated for 5 min with 10 μM camptothecin at room temperature followed by a 40-min heat shock; 5, RNA from cells heat shocked for 5 min and then treated with 10 μM camptothecin for 35 min at 36°C. The ethidium bromide-stained gel indicated that each lane contained comparable amounts of total RNA. The RNA blot was analyzed by using the *Hin*dIII-*Pst*I fragment from 88.1 (9) which hybridizes to *hsp28* and approximately 400 base pairs flanking each side of the gene.

transcription unit. In addition, those fragments whose 3′ ends were induced by camptothecin treatment (Fig. 5A, lane 3) exhibit an aberrant mobility in the absence of protease treatment, while those whose 5′ ends were induced by camptothecin treatment (Fig., 5B, lane 3) exhibit normal mobility in the absence of protease treatment.

Camptothecin induces cuts throughout the *hsp83* heat shock gene. The pattern of transcription of *hsp83* is quite different from the small heat shock genes. It is expressed at moderate levels in noninduced cells and is induced 3- to 10-fold after heat shock treatment (36). *hsp83* is a large gene containing an intron (22). Thus, we could use *hsp83* to assess whether camptothecin induces cuts in active genes in cells maintained at 24°C and also whether a transcription unit of quite different structure shows a distribution of topoisomerase I similar to that of the shorter heat shock genes. Figure 6A and B show that camptothecin induced both double- and single-stranded cuts throughout the transcribed region of *hsp83* in both noninduced and heat-shocked cells. The cuts began near the transcription start site and were present throughout the transcribed region including the intron. As with the small heat shock genes, there was a conspicuous cleavage site near the 3′ end of the gene. In addition, cutting is detected in a small transcribed gene, *T2*, located downstream of *hsp83* (36).

Figure 6C and D show that these cuts had the properties expected of a topoisomerase I cleavage site. Protein was covalently attached to the 3′ end of the fragments generated by using camptothecin since these fragments exhibited a mobility shift after proteinase K treatment (Fig. 6C, lanes 3 and 4). Fragments with 5′ ends generated by using camptothecin did not exhibit a mobility shift (Fig. 6D, lanes 3 and 4), indicating that no protein was bound to the 5′ ends.

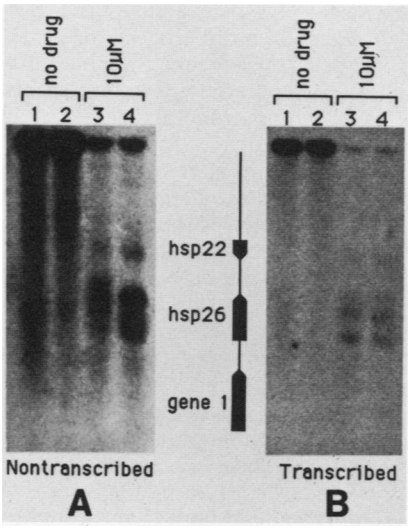

FIG. 5. Camptothecin-induced cuts near *hsp26*, *hsp22*, and gene *1*. Portions from some of the same DNA preparations used for Fig. 3 were cut with *Sal*I and run on alkaline gels. The DNA blots were analyzed by using probes 2a and 2b (Fig. 1). (A) Cuts in the nontranscribed strand of *hsp26*. Lanes: 1 and 2, heat-shocked cells not treated with camptothecin; 3 and 4 heat-shocked cells treated with 10 μM camptothecin. Lanes 1 and 3 are samples that were not treated with proteinase K. Lanes 2 and 4 are samples that were treated with proteinase K after restriction digestion and just before electrophoresis. The probe used here detects cleavage fragments whose 3′ ends were generated with camptothecin. (B) The same blot as in panel A hybridized with a probe specific for the transcribed strand of *hsp26*. This probe detects cleavage fragments whose 5′ ends were generated with camptothecin.

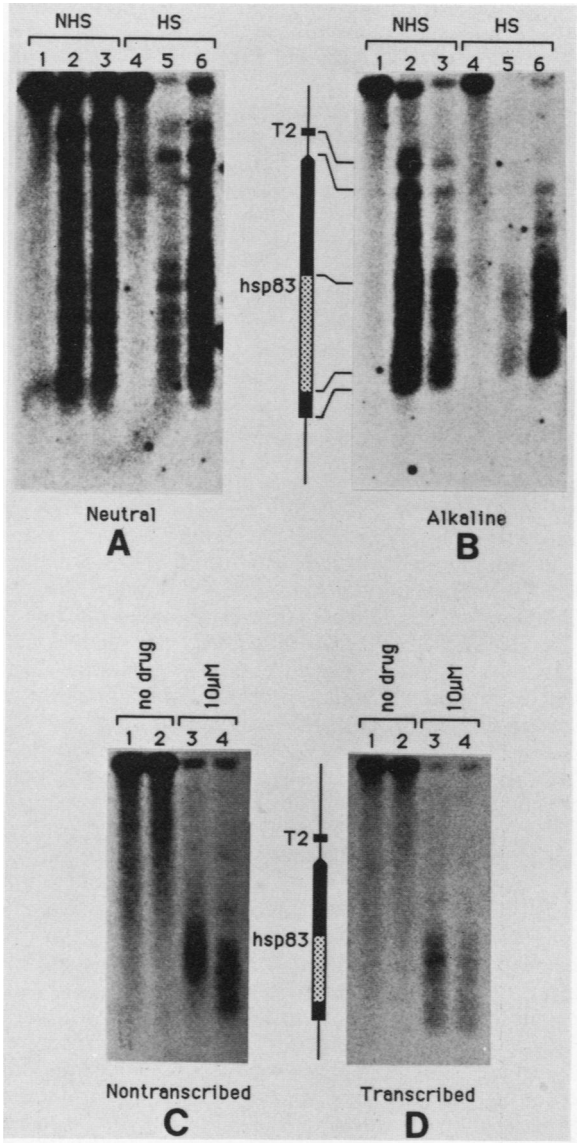

FIG. 6. Camptothecin-induced cutting at *hsp83*. (A and B) Portions of the DNA samples used in Fig. 2 were cut with *Bam*HI and run on both neutral and alkaline gels. Cleavage sites were mapped from the *Bam*HI site just upstream of *hsp83* with probe 4a (Fig. 1) which is homologous to the transcribed strand. Hence, on the alkaline gel, only cuts in the transcribed strand are detected. Lanes are as in Fig. 2. (C and D) Portions from the same DNA preparations shown in Fig. 3 were cut with *Bam*HI and run on alkaline gels. The blot was first hybridized with a probe homologous to the nontranscribed strand and then, after stripping off this probe, hybridized with a probe homologous to the transcribed strand. Lanes: 1 and 2, heat-shocked cells not treated with camptothecin; 3 and 4, heat-shocked cells treated with camptothecin. Lanes 1 and 3 are samples that were never treated with proteinase K. Lanes 2 and 4 are samples that were treated with proteinase K after restriction digestion and just before electrophoresis.

DISCUSSION

Several studies have indicated that topoisomerase I interacts with transcriptionally active regions of the chromo-

some. Immunofluorescence microscopy shows that topoisomerase I is associated with highly transcribed loci of polytene chromosomes (15, 43). Protein-DNA cross-linking shows that the interaction involves the transcribed region and not nearby flanking sequences (19). The present work shows that topoisomerase I interacts with specific sites on each strand of the DNA within the transcribed region. Moreover, very little interaction is observed outside of the transcription unit.

There can be little doubt that the camptothecin-induced cuts are due to topoisomerase I. Hsiang et al. (27) have shown that for mammalian cells, camptothecin stabilizes purified topoisomerase I but not topoisomerase II in a covalent protein-DNA intermediate. A different drug, VM26, stabilizes purified topoisomerase II, but not topoisomerase I, in a covalent protein-DNA intermediate (8). The cutting pattern induced in cells by VM26 is strikingly different from that of camptothecin, suggesting that the two drugs have very different targets. The mobility shift assay shows that a protein is tightly bound to the DNA after camptothecin treatment of cells. This is likely to be a covalent interaction as it is resistant to 0.8% SDS and to 50 mM NaOH (see Materials and Methods). Finally, the mobility shift experiment shows that the protein is associated with the 3′ end and not the 5′ end of the newly cleaved DNA. This property is diagnostic of eucaryotic topoisomerase I interaction and contrasts with topoisomerase II interaction in which the protein is covalently attached to the 5′ end of the DNA.

The double-stranded cuts observed were initially puzzling since they were not predicted by the proposed mechanism of topoisomerase I activity. Further studies looking specifically at the cleavage of each strand led us to propose that the double-stranded cuts are due to closely positioned single-stranded cuts induced by topoisomerase I on opposite strands of the DNA. Indeed, previous investigators have detected double-stranded cuts in naked DNA treated with high levels of purified topoisomerase I (31). It is very likely that high levels of this protein are associated with the heat shock genes in vivo. Immunofluorescence microscopy reveals intense staining of heat shock loci by using antibody to topoisomerase I (15). Cross-linking studies show that only a fewfold less topoisomerase I than RNA polymerase II molecules are cross-linked to the heat shock genes (19); it is well established that high levels of RNA polymerase II interact with the heat shock genes. Of course, both assays are inherently limited in their abilities to provide absolute quantitation of the level of protein associated with a gene. However, the limitations of each assay are of a different nature, and yet each assay supports the same conclusion.

The interaction of topoisomerase I with DNA corresponds very closely with transcription. For *hsp28*, three strong cuts occur within the transcribed strand of the gene, while one strong cut occurs in the other strand near the end of the gene. For the 5′ region of *hsp83*, cuts begin very near the start of transcription with no detectable cuts occurring in the flanking region upstream of the transcription start site. Despite this close correlation with transcription, camptothecin only partially inhibited transcription of *hsp28* regardless of whether it was applied before or after heat shock treatment. Moreover, similar levels of inhibition were observed at drug concentrations between 0.1 and 10 μM, indicating that the drug is probably in excess. We cannot exclude the possibility that a subpopulation of the cells is resistant to the drug. However, we favor the hypothesis that the drug merely lengthens the lifetime of the protein-DNA intermediate, slowing but not continuously blocking transcription. These intermediates are readily reversible in vitro (27). Clearly, the cuts introduced by topoisomerase I, particularly those in the transcribed strand, must be transient. Otherwise, it is hard to imagine how the transcription complex could traverse the topoisomerase I-DNA intermediate.

A comparison of the distribution of topoisomerase I cuts on several genes reveals only limited similarity. All the genes that we examined have conspicuous cuts near the 3′ ends of the transcription unit which could be related to transcription termination. In addition, cuts of various intensities occur near the transcription initiation site. The presence of cuts within the *hsp83* intron indicates a general distribution of topoisomerase I across the transcribed region. Since we failed to detect any common pattern to the cutting, we suggest that topoisomerase I interacts with the DNA in part in a manner dependent on the local DNA sequence. This is supported by the observation that purified calf thymus topoisomerase I cuts naked DNA at specific sites in the presence of camptothecin (data not shown). Based on the cutting patterns induced in vitro, potential consensus sequences have been identified (4, 5). However, a computer search for these consensus sequences within some of the regions looked at here has failed to reveal an absolute correlation between the consensus sequences and the observed camptothecin-induced cleavages (data not shown). We have found that some of the preferred cutting sites detected here in vivo are also preferred cutting sites for calf thymus topoisomerse I on the purified DNA in vitro (data not shown). Thus, we hypothesize that one important factor determining the position of topoisomerase I is the DNA sequence or structure itself. The abundance of cutting sites suggests that this recognition site occurs quite frequently. However, the mere presence of a binding site is not sufficient for the topoisomerase I to interact with the DNA as indicated by its absence from the inactive genes. The sequences must also be accessible to the enzyme. Measurements with various DNA cleavage reagents indicate that actively transcribed genes are markedly more accessible than the inactive genes in isolated nuclei (6, 50, 52).

The precise role of topoisomerase I in the transcription of chromatin remains unclear. If the active chromatin is to be maintained in a configuration under torsional strain as has been suggested (39, 45), it would seem necessary to require a very stringent regulation of the abundant topoisomerase I activity. Recently, Kmiec et al. (30) reported that while torsional strain occurs during assembly of a transcriptionally active complex in the *Xenopus* system, it is not required during transcription; the addition of topoisomerase I relaxes the supercoiled DNA without inhibiting in vitro and in vivo transcription of the *5S* gene.

Topoisomerase I, which relaxes both positively and negatively supercoiled DNA in vitro (2, 7), could fulfill the role of a general swivel to alleviate any topological constraints generated during transcription. The lack of a highly specific role is inferred from the finding that yeast cells can survive without topoisomerase I; apparently, topoisomerase II can fulfill the role of topoisomerase I in this case (21, 40, 44). Topoisomerase I might also facilitate the passage of the RNA polymerase along the chromatin fiber by facilitating nucleosome disassembly and reassembly. Germond et al. (17) reported that relatively high levels of purified topoisomerase I facilitate nucleosome assembly in vitro. It seems unlikely that the topoisomerase I would comigrate with the transcription complex; topoisomerase I and RNA

polymerase II appear to interact independently with the DNA. This is suggested by the nonrandom distribution of the topoisomerase I cleavage sites within the genes shown here. Moreover, cross-linking experiments show that different ratios of topoisomerase I to RNA polymerase II interact with different genes (19).

Clearly, much remains to be learned about the roles of topoisomerase I. We believe it to be necessary to consider the problem in the overall context of chromatin structure. Chromatin structure may restrain changes in DNA topology to very local regions, demanding a control of superhelical tension and relaxation over areas of only a few hundred base pairs. The distribution of topoisomerase I could be an important variable in the process of gene activation. Camptothecin can serve to easily and precisely locate topoisomerase I interactions in vivo and should prove to be valuable in further analysis.

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LITERATURE CITED

1. **Anderson, K. V., and J. A. Lengyl.** 1984. Histone gene expression in *Drosophila* development: multiple levels of gene regulation, p. 135–161. *In* G. S. Stein, J. L. Stein, and W. F. Marzluff (ed.), John Wiley & Sons, Inc., New York.
2. **Baase, W. A., and J. C. Wang.** 1974. An ω protein from *Drosophila melanogaster*. Biochemistry **13**:4299–4303.
3. **Berrios, M., N. Osheroff, and P. A. Fisher.** 1985. In situ localization of DNA topoisomerase II, the major polypeptide component of the *Drosophila* nuclear matrix fraction. Proc. Natl. Acad. Sci. USA **82**:4142–4146.
4. **Bonven, J. B., E. Gocke, and O. Westergaard.** 1985. A high affinity topoisomerase I binding sequence is clustered at DNase I hypersensitive sites in *Tetrahymena* R-chromatin. Cell **41**:541–551.
5. **Bullock, P., J. J. Champoux, and M. Botchan.** 1985. Association of crossover points with topoisomerase I cleavage sites: a model for nonhomologous recombination. Science **230**:954–958.
6. **Cartwright, I. L., and S. C. R. Elgin.** 1986. Nucleosomal instability and induction of new upstream protein-DNA associations accompany activation of four small heat shock protein genes in *Drosophila melanogaster*. Mol. Cell. Biol. **6**:779–791.
7. **Champoux, J. J., and R. Dulbecco.** 1972. An activity from mammalian cells that untwists supercoiled DNA—a possible swivel for DNA replication. Proc. Natl. Acad. Sci. USA **69**:143–146.
8. **Chen, G. L., L. Yang, T. C. Rowe, B. D. Halligan, K. M. Tewey, and L. F. Liu.** 1984. Nonintercalative antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. J. Biol. Chem. **259**:13560–13566.
9. **Corces, V., R. Holmgren, R. Freund, R. Morimoto, and M. Meselson.** 1980. Four heat shock proteins of *Drosophila melanogaster* coded within a 12-kilobase region in chromosome subdivision 67B. Proc. Natl. Acad. Sci. USA **77**:5390–5393.
10. **Costlow, N., and J. T. Lis.** 1984. High-resolution mapping of DNase I-hypersensitive sites of *Drosophila* heat shock genes in *Drosophila melanogaster* and *Saccharomyces cerevisiae*. Mol. Cell. Biol. **4**:1853–1863.
11. **Craig, E. A., and B. J. McCarthy.** 1980. Four *Drosophila* heat shock genes at 67B: characterization of recombinant plasmids. Nucleic Acids Res. **8**:4441–4457.
12. **DiNardo, S., K. Voekel, and R. Sternglanz.** 1984. DNA topoisomerase II mutant of *Saccharomyces cerevisiae*: topoisomerase II is required for segregation of daughter molecules at the termination of DNA replication. Proc. Natl. Acad. Sci. USA **81**:2616–2620.
13. **Earnshaw, W. C., B. Halligan, C. A. Cooke, M. M. S. Heck, and L. F. Liu.** 1985. Topoisomerase II is a component of mitotic chromosome scaffolds. J. Cell Biol. **100**:1706–1715.
14. **Fisher, L. M.** 1984. DNA supercoiling and gene expression. Nature (London) **307**:686–687.
15. **Fleishmann, G., G. Pflugfelder, E. K. Steiner, K. Javaherian, G. C. Howard, J. C. Wang, and S. C. R. Elgin.** 1984. *Drosophila* DNA topoisomerase I is associated with transcriptionally active regions of the genome. Proc. Natl. Acad. Sci. USA **81**:6958–6962.
16. **Gellert, M.** 1981. DNA topoisomerase. Annu. Rev. Biochem. **50**:879–910.
17. **Germond, J. E., J. Rouieve-Yaniv, M. Yaniv, and D. Brutlag.** 1979. Nicking-closing enzyme assembles nucleosome-like structures in vitro. Proc. Natl. Acad. Sci. USA **76**:3779–3783.
18. **Gilmour, D. S., J. T. Lis.** 1985. In vivo interactions of RNA polymerase II with genes of *Drosophila melanogaster*. Mol. Cell. Biol. **5**:2009–2018.
19. **Gilmour, D. S., G. Pflugfelder, J. C. Wang, and J. T. Lis.** 1986. Topoisomerase I interacts with transcribed regions in *Drosophila* cells. Cell **44**:401–407.
20. **Gocke, E., B. J. Bonven, and O. Westergaard.** 1983. A site and strand specific nuclease activity with analogies to topoisomerase I frames the rRNA gene of *Tetrahymena*. Nucleic Acids Res. **11**:7661–7678.
21. **Goto, T., and J. C. Wang.** 1985. Cloning of yeast *TOP1*, the gene encoding topoisomerase I, and the construction of mutants defective in both DNA topoisomerase I and DNA topoisomerase II. Proc. Natl. Acad. Sci. USA **82**:7178–7182.
22. **Hackett, R. W., and J. T. Lis.** 1983. Localization of the hsp83 transcript within a 3292 nucleotide sequence from the 63B heat shock locus of *D. melanogaster*. Nucleic Acids Res. **11**:7011–7030.
23. **Han, S., A. Udvardy, and P. Schedl.** 1985. Novobiocin blocks the *Drosophila* heat shock response. J. Mol. Biol. **183**:13–29.
24. **Harland, R. M., H. Weintraub, and S. L. McKnight.** 1983. Transcription of DNA injected into *Xenopus* oocytes is influenced by template topology. Nature (London) **302**:38–43.
25. **Heller, R. A., E. R. Shelton, V. Dietrich, S. C. R. Elgin, and D. L. Brutlag.** 1986. Multiple forms and cellular localization of *Drosophila* DNA topoisomerase II. J. Biol. Chem. **261**: 8063–8069.
26. **Holm, C., T. Goto, J. C. Wang, and D. Botstein.** 1985. DNA topoisomerase II is required at the time of mitosis in yeast. Cell **41**:553–563.
27. **Hsiang, Y.-H., R. Hertzberg, S. Hecht, and L. F. Liu.** 1985. Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. J. Biol. Chem. **260**:14873–14878.
28. **Jackson, D. A., and P. R. Cook.** 1985. Transcription occurs at a nucleoskeleton. EMBO J. **4**:919–925.
29. **Javaherian, K., Y.-C. Tse, and J. Vega.** 1982. *Drosophila* topoisomerase I: isolation, purification, and characterization. Nucleic Acids Res. **10**:6945–6955.
30. **Kmiec, E. B., R. Masaru, and A. Worcel.** 1986. Gyration is required for 5S RNA transcription from a chromatin template. Proc. Natl. Acad. Sci. USA **83**:1305–1309.
31. **Liu, L. F., T. C. Rowe, L. Yang, K. M. Tewey, and G. L. Chen.** 1983. Cleavage of DNA by mammalian DNA topoisomerase II. J. Biol. Chem. **258**:15363–15379.
32. **Luchnik, A. N., V. V. Bakayev, I. B. Zbarsky, and G. P. Georgiev.** 1982. Elastic torsional strain in DNA within a fraction of SV40 minichromosomes: relation to transcriptionally active chromatin. EMBO J. **1**:1353–1358.
33. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning; a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor. N. Y.
34. **Messing, J.** 1983. New M13 vectors for cloning. Methods

Enzymol. **101**:20–78.
35. **Nedospasov, S. A., and G. P. Georgiev.** 1980. Non-random cleavage of SV40 DNA in the compact minichromosome and free in solution by micrococcal nuclease. Biochem. Biophys. Res. Commun. **92**:532–539.
36. **O'Connor, D., and J. T. Lis.** 1981. Two closely linked transcription units within the 63B heat shock puff locus of *D. melanogaster* display strikingly different regulation. Nucleic Acids Res. **9**:5075–5092
37. **Robinson, S. I., B. D. Nelkin, and B. Vogelstein.** 1982. The ovalbumin gene is associated with the nuclear matrix of chicken oviduct cells. Cell **28**:99–106.
38. **Rowe, T. C., J. C. Wang, and L. F. Liu.** 1986. In vivo localization of DNA topoisomerase II cleavage sites on *Drosophila* heat shock chromatin. Mol. Cell. Biol. **6**:985–992.
39. **Ryoji, M., and A. Worcel.** 1984. Chromatin assembly in *Xenopus* oocytes: in vivo studies. Cell **37**:21–32
40. **Saavedra, R. A., and R. H. Huberman.** 1986. Both DNA topoisomerase I and II relax 2 μ plasmid DNA in living yeast cells. Cell **45**:65–70.
41. **Sirotkin, K., and N. Davidson.** 1982. Developmentally regulated transcription from *Drosophila melanogaster* chromosome site 67B. Dev. Biol. **89**:196–210.
42. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
43. **Steiner, E. K., J. C. Eissenberg, and S. C. R. Elgin.** 1984. A cytological approach to the ordering of events in gene activation using the *Sgs-4* locus of *Drosophila melanogaster*. J. Cell Biol. **99**:233–238.
44. **Uemura, T., and M. Yanagida.** 1984. Isolation of type I and II DNA topoisomerase mutants from fission yeast: single and double mutants show different phenotypes in cell growth and chromatin organization. EMBO J. **3**:1737–1744.
45. **Villeponteau, B., M. Landell, and H. Martinson.** 1984. Torsional stress promotes the DNAase I sensitivity of active genes. Cell **39**:469–478.
46. **Vosberg, P.-H.** 1985. DNA topoisomerase: enzymes that control DNA conformation. Curr. Top. Microbiol. Immunol. **114**: 19–102.
47. **Wahl, G. M., M. Stern, and G. M. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. USA **76**:3683–3687.
48. **Wang, J. C.** 1973. Protein ω: a DNA swivelase from *Escherichia coli*?, p. 163–174. *In* R. D. Wells and R. B. Inman (ed.), *In* DNA synthesis in vitro. University Park Press, Baltimore.
49. **Wang, J. C.** 1985. Topoisomerase. Annu. Rev. Genet. **54**: 665–698.
50. **Weintraub, H., and M. Groudine.** 1976. Chromosomal subunits in active genes have an altered conformation. Science **193**: 848–856.
51. **Wu, C.** 1980. The 5′ ends of *Drosophila* heat shock genes in chromatin are hypersensitive to DNase I. Nature (London) **286**:854–860.
52. **Wu, C., Y.-C. Wong, and S. C. R. Elgin.** 1979. The chromatin structure of specific genes. II. Disruption of chromatin structure during gene activity. Cell **16**:807–814.