Volume 17 Number 21 1989 Nucleic Acids Research

Interaction of topoisomerase 1 with the transcribed region of the *Drosophila* HSP 70 heat shock gene

Paul E.Kroeger and Thomas C.Rowe*

Department of Pharmacology and Therapeutics, University of Florida College of Medicine, Gainesville, FL 32610, USA

Received August 4, 1989; Revised and Accepted October 4, 1989

ABSTRACT

Topoisomerase I cleavage sites have been mapped *in vivo* on the Hsp70 heat shock gene of *Drosophila melanogaster* cells using the drug camptothecin. Topoisomerase I cleavage was only observed when the Hsp70 gene was transcriptionally active. Site-specific single-strand DNA cleavage by topoisomerase I was confined to the transcribed region of the Hsp70 gene and occurred on both the transcribed and nontranscribed DNA strands. A number of the single-strand breaks on the complementary DNA strands occurred in close proximity giving rise to double-stranded DNA breaks. Inhibition of heat-induced Hsp70 transcription by either Actinomycin D (Act D) or 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) inhibited topoisomerase I cleavage except at the 5′ and to a lesser extent the 3′ end of the gene. Camptothecin (100 μM) inhibited transcription of the Hsp70 gene greater than 95%. These results suggest that topoisomerase I is intimately associated with and has an integral part in Hsp70 gene transcription.

INTRODUCTION

DNA topoisomerases, type I and II, catalyze changes in the topological structure of DNA by breaking and rejoining either one or both strands of the DNA duplex, respectively (reviewed in 1−3). In prokaryotes, topoisomerase mediated changes in DNA supercoiling have been shown to affect gene expression (4,5). Evidence suggests that DNA topology also plays a critical role in regulating eukaryotic gene expression. Experiments in a Yeast mutant with temperature sensitive mutations in both topoisomerase I and II demonstrated that ribosomal and poly (A) RNA synthesis were significantly inhibited when these cells were grown at the restrictive temperature (6). This finding suggested that topoisomerases acted as a swivel in the DNA to release the torsional stress that was associated with transcription. A role of topoisomerase I in transcription has also been suggested by studies in *Chironomus tentans* salivary gland cells. Synthesis of rRNA and Balbiani ring RNA in *Chironomus tentans* was arrested by microinjection of anti-topoisomerase I antibody into the nuclei of these cells. Inhibition was reversed upon addition of exogenous topoisomerase I suggesting that this enzyme was an essential component in the transcriptional process (7). More recent experiments have suggested that translocation of RNA polymerase along a gene generates positive supercoils ahead and negative supercoils behind the moving transcription complex (8−10, reviewed in 11). In bacteria, the negative and positive supercoils are removed by topoisomerase I and topoisomerase II, respectively. Studies in Yeast suggest that topoisomerase I and II perform a similar role in eukaryotic transcription (9,12).

The Hsp70 heat shock genes in *Drosophila* provide an excellent system for studying the role of topoisomerases in regulating gene expression (13−15). These genes are highly

© IRL Press

conserved throughout evolution and can be transcriptionally activated by heat (reviewed in 16). There is also a great deal of information available concerning the structure and regulation of these genes. In this paper we have investigated the interaction of topoisomerase I with the Hsp70 genes in cultured *Drosophila* K_c cells before and after heat-induced activation of transcription using the topoisomerase I inhibitor camptothecin. This drug interferes with the normal DNA breakage and reunion reaction of this enzyme by stabilizing a covalent enzyme-DNA intermediate termed the cleavable complex (17). Treatment of this complex with a protein denaturant (i e., SDS) results in the formation of single-stranded protein-linked DNA breaks. These DNA break sites can readily be mapped on specific cellular genes using Southern hybridization techniques (15). The location of these breaks represents a footprint of topoisomerase I-active sites on chromatin in cells. This approach has been effectively used by other investigators to study the interaction of topoisomerase I with a variety of cellular genes including the small heat-shock genes in Drosophila (14), the rat tyrosine aminotransferase gene (18), and the ribosomal genes in human, *X. laevis*, and *Dictyostelium* cells (19−21). These studies showed that topoisomerase I is localized within the transcribed regions of these genes. In the case of the small heat shock genes in *Drosophila*, topoisomerase I cleavage could only be observed following activation of these genes by heat suggesting that this enzyme played an active role in transcription (14). Our results on the Hsp70 gene in *Drosophila* are consistent with these earlier findings. Furthermore, we find that inhibition of Hsp70 transcription by Act D or DRB (22,23) results in the loss of most of the topoisomerase I binding sites on the gene suggesting that topoisomerase I binding to the Hsp70 DNA is primarily in response to changes in the structure of the Hsp70 gene caused by the movement of the RNA polymerase complex.

MATERIALS AND METHODS

Enzymes, Nucleic acids and Drugs

Restriction endonucleases were obtained from Boehringer-Mannheim (Indianapolis, IN). Materials necessary for nick-translation were purchased from BRL (Gaithersburg, MD). Camptothecin was obtained from the National Cancer Institute and prepared as a 100 mM stock in DMSO and stored frozen at −20°C until use. The DNA clones used in these studies were prepared by standard cloning techniques and are derivatives of the original *Drosophila* Hsp70 clone pPW229 (24). The clone pMR2 was constructed by inserting the 1.1 kb Pst I-Pst I fragment spanning the 5′ half of the Hsp70 gene into the Pst I site of the riboprobe vector pGEM 2 (Promega Biotech). The clone pMR4 contains the 610 bp BamHI-XbaI fragment spanning the 3′ region of the Hsp70 gene that has been inserted at the Bam HI site of pGEM 2.

Cell culture

Drosophila K_c cells were obtained from Dr. Neil Osheroff (Vanderbilt University). The cells were maintained at 25°C in D-22 medium supplemented with penicillin and streptomycin as described previously (25). Heat shock treatments were performed in a water bath at 37°C.

Isolation of Genomic DNA

Cellular DNA was purified from cultured *Drosophila* Kc cells as described before (15). *Drosophila* Kc cells (5 ml) at $5-10\times10^6$ cells/ml were quickly harvested by centrifugation and the resulting cell pellet resuspended in 1 ml lysis buffer (20 mM Tris-HCl, pH 8.0, 1% SDS). Proteinase K and EDTA were added to final concentrations of 500 μg/ml and 20 mM respectively. After incubating for 16 hrs. at 37°C, the lysates were phenol extracted

3 times and then ether extracted once. RNaseA was then added to a final concentration of 20 μg/ml and the samples incubated an additional hour at 37°C. The samples were then phenol and ether extracted one time before precipitating the DNA with ethanol at −20°C overnight. The resulting DNA precipitates were collected by centrifugation and digested with either Bam HI or Xba I using the reaction conditions recommended by the manufacturer.

Isolation of Cellular RNA

Total cellular RNA was prepared essentially as described by Chomczynski and Sacchi (26). Briefly, cells were harvested as described above and lysed in 500 μl of Solution A (4M Guanidine Isothiocyanate, 10 mM Tris-HCl, pH 7.5, 0.5% Sarkosyl, 0.1 M 2-mercaptoethanol). To the lysate the following solutions were added sequentially with mixing: 0.1 vol. 2 M NaOAc (pH 5.2), 1 vol. phenol, 0.2 vol. chloroform/isoamyl alcohol (24:1). The mixture was cooled on ice and then centrifuged at 10,000× g for 15 min. at 4°C. The upper aquaeous phase was removed and precipitated with 1 vol. of isopropanol at −20°C for 1 hr. The RNA was recovered, dissolved in Solution A, and reprecipitated. After centrifugation, the RNA pellet was dissolved in DEPC treated water and stored at −70°C.

Gel Electrophoresis.

Neutral agarose gel electrophoresis-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, 0.1% SDS) and electrophoresed in a 1.4% horizontal agarose gel containing 40 mM Tris base, 90 mM boric acid, 2 mM EDTA, pH 8.3 for 16 h at 70 V.

Alkaline agarose gel electrophoresis-DNA (10 μg) which had been digested with restriction enzymes was ethanol precipitated and resuspended in 10 μl of a solution containing 50 mM NaOH, 1 mM EDTA, 2.5% Ficoll, 0.025% bromocresol green. The DNA was then electrophoresed in a 1.4% vertical agarose gel containing 30 mM NaOH, 1 mM EDTA for 16 hrs. at 70V using a Hoefer vertical gel apparatus (Hoefer Sci. Inst., San Francisco, CA).

Formaldehyde agarose gel electrophoresis-RNA samples (5 μg) were electrophoresed in a 1.4% horizontal agarose gel containing 40 mM morpholinopropanesulfonic acid, pH 7.0, 50 mM sodium acetate, 5 mM EDTA, 2.2 M formaldehyde at 75 V for 2 h as described by Maniatis et al.(27)

DNA Transfer and Hybridization

DNA was transferred from neutral or alkaline agarose gels onto nitrocellulose filter paper (BA 85, Schleicher and Schuell, Keene, N.H.) as described by Maniatis et al. (27). The filters were then preincubated in hybridization buffer (5× SSPE, 0.1% SDS, 0.25% non-fat dry milk) at 68°C. After 4 h, the filters were placed in fresh hybridization buffer containing [^{32}P]-labeled probe and incubated at 68°C for an additional 20 hrs. The filters were then washed in 0.1 M KPO_4, pH 7.0 for 30 min. and then in 1× SSC (0.15 M NaCl, 15 mM NaCitrate, pH 7.0), 0.5 % SDS for an additional 30 min. at room temperature. A final wash was then done in 0.1× SSC, 0.5 % SDS at 65°C for 30 min. DNA blots that were hybridized to riboprobes were subjected to an addtional wash in 0.3M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1μg/ml RNase A for 30 min. at 37°C.

RNA Transfer and Hybridization

RNA was transferred from formaldahyde gels onto nitrocellulose filter paper as described by Maniatis et al. (27). The filters were preincubated in the RNA hybridization buffer (5×SSPE, 1.0% SDS, 50% formamide, 0.25% non-fat dry milk) for 4 h at 42°C. The

filters were then placed in fresh RNA hybridization buffer containing [^{32}P]-labeled probe and incubated an additional 20 h at 42°C. The filters were subsequently washed according to the procedure used for the DNA filters.

Labeling of DNA and RNA Probes

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

Riboprobes-Strand specific RNA probes were prepared as described by Melton et al. (28) and all reagents were obtained from Promega Biotech (Madison, WI). Specifically, 2 μg of either HindIII (SP6 reactions) or BamHI (T7 reactions) linearized pMR4 was incubated in 20 μl transcription buffer (40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, and 10 mM NaCl, 10 mM DTT, 1 unit/μl RNasin, 12 μM CTP, and 500 μM ATP, GTP, and UTP) containing 100 μCi [α-^{32}P]-CTP (3000 Ci/mMole) and 20 units of either Sp6 or T7 RNA polymerase. The Sp6 and T7 reactions were incubated at 40°C and 37°C respectively for 90 min. RNase free DNase was then added to 1 unit/μg of template DNA and the incubation continued at 37°C for an additional 15 min. The reaction was then stopped by adding EDTA to 20 mM. Unincorporated [α-^{32}P]-CTP was removed by ethanol precipitating the RNA in the presence of 2.5 M ammonium acetate several times.

Autoradiography

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

RESULTS

Camptothecin induced cleavage of the Hsp70 transcribed region by topoisomerase I

The *Drosophila* Hsp70 gene provides an ideal model system for the characterization of topoisomerase I function in the regulation of eukaryotic transcription as this gene is inducible and regulated very tightly at the transcriptional level (16). *Drosophila* cells contain from 5−6 copies of the Hsp 70 gene which are located at the 87A and 87C loci on chromosome 3 (29,30). These genes are highly conserved and consist of a 5′, 0.35 kb regulatory element and a 2.2 kb transcribed region as is illustrated in Fig. 1. Normally, the Hsp70 genes are transcriptionally silent. However, transcription is rapidly activated to high levels upon elevating the growth temperature from 25°C to 37°C (16). The association of topoisomerase I with this gene was initially investigated in cultured *Drosophila* K_c cells incubated at either 25°C or 37°C in the presence of 20μM camptothecin (Fig. 2). The DNA was isolated from the treated cells, digested with XbaI or Bam HI, and electrophoresed through a denaturing 1.4% alkaline agarose gel. The gel was then blotted onto a nitrocellulose filter and hybridized to nick-translated pMR4 probe (see map of Hsp70 gene in Fig. 1). In this way, drug-induced DNA topoisomerase cleavage sites could readily be mapped relative to the Xba I or Bam HI restriction sites of the Hsp70 gene using the indirect end-labeling procedure (31). At 25°C there was no detectable drug-induced topoisomerase I cleavage throughout the 5′ (lane 1) or 3′ (lane 3) region in the Hsp70 gene. However, at 37°C, there was extensive camptothecin induced cleavage throughout the transcribed region of the gene at specific and reproducible sites (lanes 2 and 4). Little detectable cleavage was observed in the 5′- or 3′-flanking nontranscribed regions of the Hsp70 gene. The exception

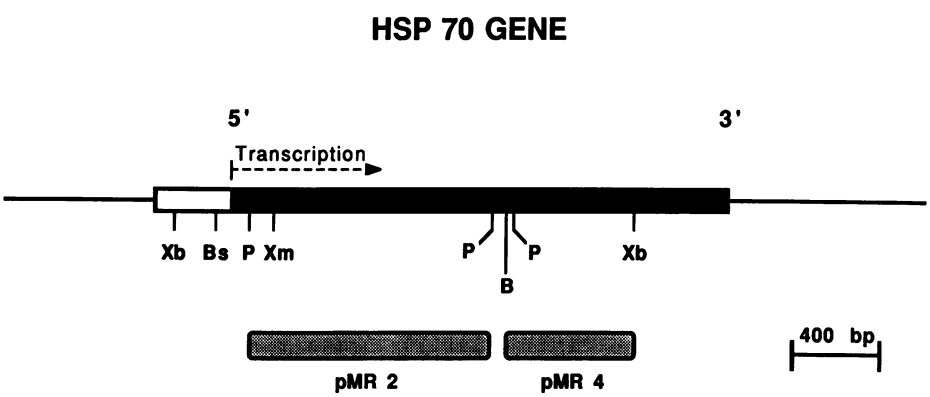


Figure 1. *Map of the Drosophila Hsp70 gene.* The *Drosophila* Hsp70 gene consists of a 360 bp nontranscribed regulatory region (open box) adjoining the 2.2 kbp transcribed region (solid box). The shaded boxes indicate the cloned regions of the Hsp70 gene which were used in the Southern and Northern blotting experiments. Abbreviations for restriction sites are: B, BamHI; Bs, BssHII; P, Pst I; Xb, Xba I; Xm, XmnI. See text for additional details.

was a site that occurred in the nontranscribed regulatory region approximately 150 to 200 bases uptstream from the start of transcription. These results are schematically summarized in Fig. 2B and are similar to those found for the small Hsp genes in *Drosophila* (14) as well as the human, *Xenopus* and *Dictyostelium* ribosomal RNA genes (19−21) and the rat tyrosine aminotransferase gene (18).

Detection of strand-specific DNA breaks on the active and inactive Hsp70 gene

To ascertain whether topoisomerase I cleavage was occurring on one or both strands of the activated Hsp70 gene, Southern blots of XbaI-restricted DNA from drug treated cells were hybridized to strand-specific riboprobes made from pMR4 (Fig 3A). RNA probes hybridizing to the transcribed (lanes 1−3) or nontranscribed (lanes 4−6) strands of the Hsp70 DNA were prepared using SP6 or T7 RNA polymerases, respectively (28). The results of this experiment demonstrate that distinct patterns of topoisomerase I cleavage are detected on the transcribed (lane 3) and nontranscribed (lane 4) strands of the Hsp70 gene. Although the cleavage sites on both DNA strands were evenly distributed throughout the transcribed region of the gene, the majority of cleavage was localized to the transcribed strand (compare lanes 3 and 4). A similar preferential cleavage of the transcribed strand in the presence of campthothecin has also been observed for the small Hsp genes in *Drosophila* as well as for the rat tyrosine aminotransferase gene and the *Dictyostelium* rRNA genes (14,18,21).

There is evidence that topoisomerase I plays an important role in removing supercoils which are generated in DNA regions undergoing transcription (6−12). This might explain the exclusive association of topoisomerase I with actively transcribed genes. If topoisomerase I binding to the Hsp70 gene is stimulated by movement of RNA polymerase along the DNA template, then binding should be abolished by drugs which inhibit transcription. Act D and the adenosine analogue DRB are both potent inhibitors of RNA polymerase II catalyzed transcription (22,23). As is evident from Fig. 3B, preincubation of *Drosophila* K_c cells for 5 min with 1 μM Act D or 65 μM DRB at 25°C completely blocks heat-induced Hsp70 RNA synthesis (compare lanes 1,4,and 5). These drug concentrations also

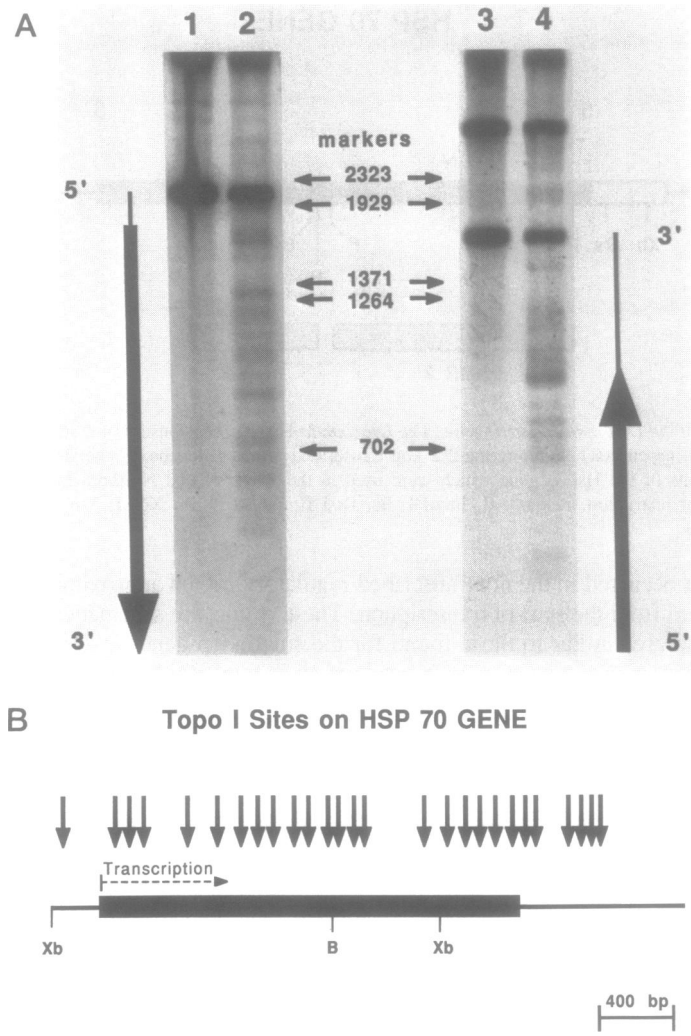


Figure 2. *Camptothecin -induced topoisomerase I cleavage sites on the Hsp70 gene before and after heat shock.* (A) *Drosophila* Kc cells which had been incubated 15 min at either 25°C (lanes 1 and 3) or 37°C (lanes 2 and 4) were treated with camptothecin (20μM) for an additional 10 min at these temperatures and the cells then lysed with SDS as described in MATERIALS AND METHODS. The DNA isolated from these cells was restricted with either Xba I (lanes 1 and 2) or Bam HI (lanes 3 and 4) before electrophoresis through a 1.4% alkaline agarose gel to map the cleavage sites in the 5′ or 3′ regions of the Hsp70 gene respectively. The Southern blot of this gel was then hybridized to nick-translated pMR4 probe. The DNA markers are given in base pairs. (B) The solid arrows represent the positions of the topoisomerase I cleavage sites on the Hsp70 gene as determined from the Southern blot in Panel A. The letters B and Xb correspond to Bam HI and Xba I restriction sites respectively.

cause a corresponding decrease in topoisomerase I mediated cleavage of the transcribed and nontranscribed strands of the Hsp70 DNA suggesting that ongoing transcription by RNA polymerase II stimulates topoisomerase binding to the Hsp70 gene (Fig. 3A, lanes

1,2,5, and 6). Not all of the camptothecin-induced DNA cleavage by topoisomerase I was abolished when transcription was blocked by Act D or DRB. Cleavage sites at the 5'-end of the Hsp70 gene between +50 and +150, relative to the start of transcription, were still present at concentrations of Act D or DRB which appeared to completely block Hsp70 RNA synthesis. Act D has recently been reported to stabilize a topoisomerase I-DNA cleavable complex *in vitro* (32). However, the cleavage observed in the presence of Act D was not due to a direct action of this drug on topoisomerase I since neither Act D or DRB caused any detectable cleavage of the Hsp70 DNA in the absence of camptothecin at either 25°C or 37°C (data not shown).

Camptothecin induces double strand breaks on the Hsp70 gene

Although camptothecin stabilizes the formation of single-stranded DNA breaks by topoisomerase I *in vitro*, *in vivo* studies have demonstrated the presence of double- as well as single-stranded DNA breaks in cells treated with camptothecin (14,19). The double-strand breaks apparently result from closely spaced enzyme-mediated single-strand breaks on the opposing DNA strands. Consistent with these observations, a number of the single-strand cleavage sites present on the complementary strands of the Hsp70 DNA are coincident and could give rise to double-stranded DNA breaks (Fig. 3, compare lanes 3 and 4). To determine if closely spaced breaks on opposite strands of the Hsp70 gene could result in double-stranded DNA breaks, we analyzed DNA from cells treated with camptothecin on nondenaturing agarose gels (Fig. 4). Prior to electrophoresis, the DNA was digested with either Xba I or Bam HI so that double-strand breaks at either the 5' and 3' ends of the gene could be detected when the blots were hybridized to nick-translated pMR4 probe. As is evident in Figure 4, double-stranded DNA cleavage was detected throughout the transcribed region of the Hsp70 gene (lanes 4 and 8) suggesting that juxtaposition of single-strand topoisomerase I sites on the complementary strands of the Hsp70 DNA results in double-strand DNA breaks. Although camptothecin-induced double-stranded DNA cleavage at a number of sites was significantly inhibited by either Act D and DRB, sites present at the 5'-end and to a lesser extent at the 3'-end of the gene were not abolished (lanes 1,2, 5, and 6). In fact, cleavage sites located between +50 to +150, relative to the start site of transcription, appeared to be stronger in cells which had been treated with either Act D or DRB. This result initially puzzled us. However, Northern blotting of RNA following electrophoresis through 4% polyacrylamide-urea gels has revealed the presence of small Hsp70 transcripts (100−200 nucleotides in length) in heat-shocked cells treated with Act D or DRB. These transcripts hybridized to the BssHII-XmnI fragment of the Hsp70 gene (−68 to +200) indicating that these transcripts are generated near the 5'-end of the gene (data not shown). This result suggests that Hsp70 transcription was initiated but then prematurely terminated near the 5'-end of the gene by these drugs. These results are consistent with recent findings from other laboratories that also indicate Act D and DRB inhibit RNA synthesis by causing premature termination of initiated transcripts (23,33,34). This may explain why Act D or DRB does not abolish topoisomerase I cleavage at the 5'-end of the Hsp70 gene. However, these results cannot explain the residual topoisomerase I cleavage observed at the 3'-end of the Hsp70 gene following inhibition of Hsp70 transcription by Act D or DRB.

Attenuation of topoisomerase I cleavage by DRB

To further investigate the relationship between topoisomerase I binding and transcription, we added DRB to cells shortly after heat-induced activation of Hsp70 RNA synthesis and then monitored the loss of camptothecin-induced topoisomerase I cleavage from the Hsp70

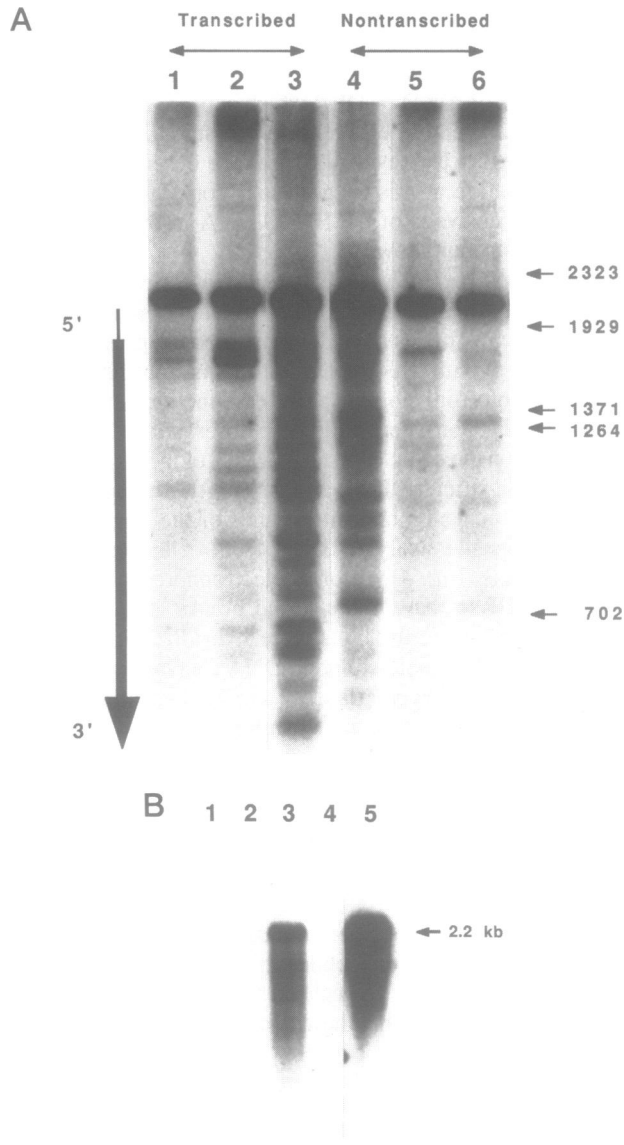


Figure 3. *Topoisomerase I DNA breaks on the transcribed and nontranscribed strands of the Hsp70 gene in the presence of Act D or DRB.* (A) *Drosophila* K_c cells were treated with either Act D or DRB for 5 min. at 25°C and then shifted to 37°C for an additional 25 min. The final 15 min of the incubation was done in the presence of 20μM camptothecin. The DNA was purified, restricted with XbaI and then electrophoresed through duplicate 1.4 % alkaline agarose gels. Following electrophoresis the gels were blotted onto nitrocellulose and hybridized to [^{32}P]-labeled riboprobes made from the plasmid pMR4 using either SP6 or T7 RNA polymerases

DNA (Fig. 5). Cleavage in the 5′ and 3′ regions of the Hsp70 DNA was determined by hybridizing Bam HI restricted DNA to either pMR2 or pMR 4 probes respectively (see schematic in Fig. 1). Cells were heat-shocked for 10 min and then DRB (65 μM) was added to arrest ongoing Hsp70 transcription. At 0, 3, 5, 10, and 20 min after the addition of DRB, cells were treated for 1 min with 100 μM camptothecin and then immediately lysed. As is apparent from Panel A in Fig 5, a majority of the topoisomerase I cleavage in the 5′ region of the gene was abolished within 1 min after DRB treatment. As was previously shown in Fig. 3A, the only topoisomerase I sites remaining occurred just downstream from the start site of transcription between nucleotides +50 to +150. Cleavage at these sites remained even after 20 min following the addition of DRB (lane 8). When we analyzed the 3′ region of the Hsp70 gene, topoisomerase cleavage could still be detected after 1 min of DRB treatment (Fig. 5B, lane 4). However, by 3 min. most of these sites were gone with the exception of several sites which flanked the 3′ end of the gene (lane 5). Similar to the residual topoisomerase I cleavage at the 5′-end, these sites were detectable for up to 20 min following DRB treatment (lane 8).

Inhibition of Hsp70 Transcription by Camptothecin

Camptothecin has been shown to significantly inhibit total cellular RNA synthesis in HeLa cells greater than 50% at 5 μM (35). To investigate the affect of camptothecin on Hsp70 transcription in *Drosophila* we measured the amount of Hsp70 RNA present in cells following a 15 min heat shock done in the presence or absence of 2, 20, or 100 μM drug. There is significant inhibition of heat-induced Hsp70 transcription at 20 and 100 μM camptothecin (compare lanes 2 and 3 with lane 7). Densitometry scans of the autoradiograph indicate that camptothecin inhibits transcription greater than 95% at 100 μM. In contrast, the topoisomerase II inhibitor VM26 (36) only slightly inhibited Hsp70 transcription (lanes 4−6). Even at 100 μM VM26 transcription was inhibited less than 15%.

DISCUSSION

Previous studies have suggested that topoisomerase I is localized to regions of chromatin undergoing active transcription. Immunofluorescent staining experments in *Drosophila* polytene chromosomes with topoisomerse I specific antibodies showed that topoisomerase I was preferentially associated with regions of chromatin which were being actively expressed (37). These results have been further corroborated by recent photo cross-linking experiments in *Drosophila* which have also shown that topoisomerase I has a high affinity for regions of chromatin which are being transcribed (13). An association of topoisomerase I with active chromatin has also been suggested by *in vitro* studies which have demonstrated that topoisomerase I activity was high in nucleosomes isolated from transcriptionally active but not inactive chromatin (38).

We have investigated the interaction of topoisomerase I with Hsp 70 chromatin using the inhibitor camptothecin. This drug interferes with the DNA breakage-reunion reaction catalyzed by topoisomerase I by stabilizing an intermediate called the cleavable complex

as described in MATERIALS AND METHODS. The SP6 and T7 probes hybridized to the transcribed and nontranscribed strands of the Hsp70 gene respectively. Lanes 1−3: topoisomerase I cleavage sites on the transcribed strand; Lanes 4−6, topoisomerase cleavage sites on the nontranscribed strand. Lanes 1 and 5, 4μM Act D; Lanes 2 and 6, 65μM DRB; Lanes 3 and 4 camptothecin alone. Arrows at the right side of the figure represent position of DNA markers (given in bases). (B) *Drosophila* K_c cells were treated as in 3A and the RNA was isolated and analyzed by Northern blotting as described in MATERIALS AND METHODS. Lanes 1 and 2, 1 and 5 μM Act D; Lanes 3 and 4, 6.5 and 65 μM DRB; Lane 5, 37°C heat-shock control.

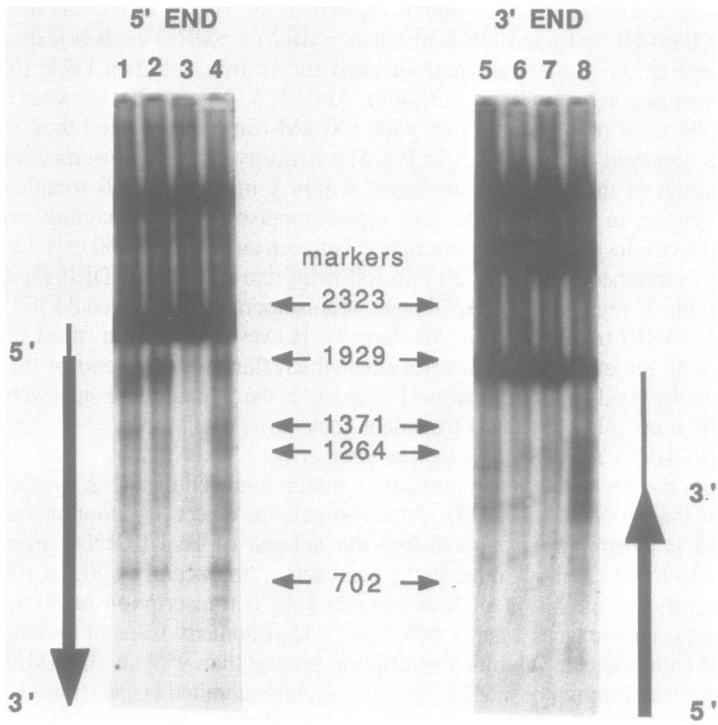


Figure 4. *Camptothecin induces double strand breaks on the Hsp70 gene.* DNA was purified from cells that had been treated with either Act D or DRB for 5 min at 25°C before shifting the cells to 37°C for an additional 25 min in the presence or absence of the inhibitors as described in MATERIALS AND METHODS. The final 15 min. of the 37°C incubations were done in the presence 20 μM camptothecin to induce topoisomerase I cleavage. The DNA was restricted with either XbaI (lanes 1−4) or BamHI (lanes 5−8) and electrophoresed in a 1.4% neutral agarose gel. The DNA was then transferred to nitrocellulose and probed with nick-translated pMR4 probe. Lanes 1 and 5, cells treated with 65 μM DRB; Lanes 2 and 6, cells treated with 1 μM Act D; Lanes 3 and 7, cells incubated at 25°C in the presence of camptothecin; Lanes 4 and 8, cells incubated at 37°C for 15 min before adding 20 μM camptothecin for an additional 10 min at 37°C.

(17). Treatment of this complex with a protein denaturant results in single-strand protein-linked DNA breaks with topoisomerase I covalently linked to the 3'-ends of the broken DNA strands. We have mapped the camptothecin-induced topoisomerase I cleavage sites on Hsp 70 chromatin in *Drosophila* K_c cells before and after heat-induced activation of Hsp70 transcription. Topoisomerase cleavage of the Hsp70 gene was localized within the transcribed region and could only be detected following heat-induced activation of Hsp70 transcription (Fig. 1). The localization of camptothecin-induced topoisomerase cleavage to transcriptionally active segments of cellular DNA has also been observed for the small Hsp genes in *Drosophila* (14), the rat tyrosine amino transferase gene (18) and the ribosomal genes of several different organisms (19−21).

Evidence has recently suggested that the movement of a RNA polymerase complex along a gene can generate a wave of positive supercoils ahead and negative supercoils behind

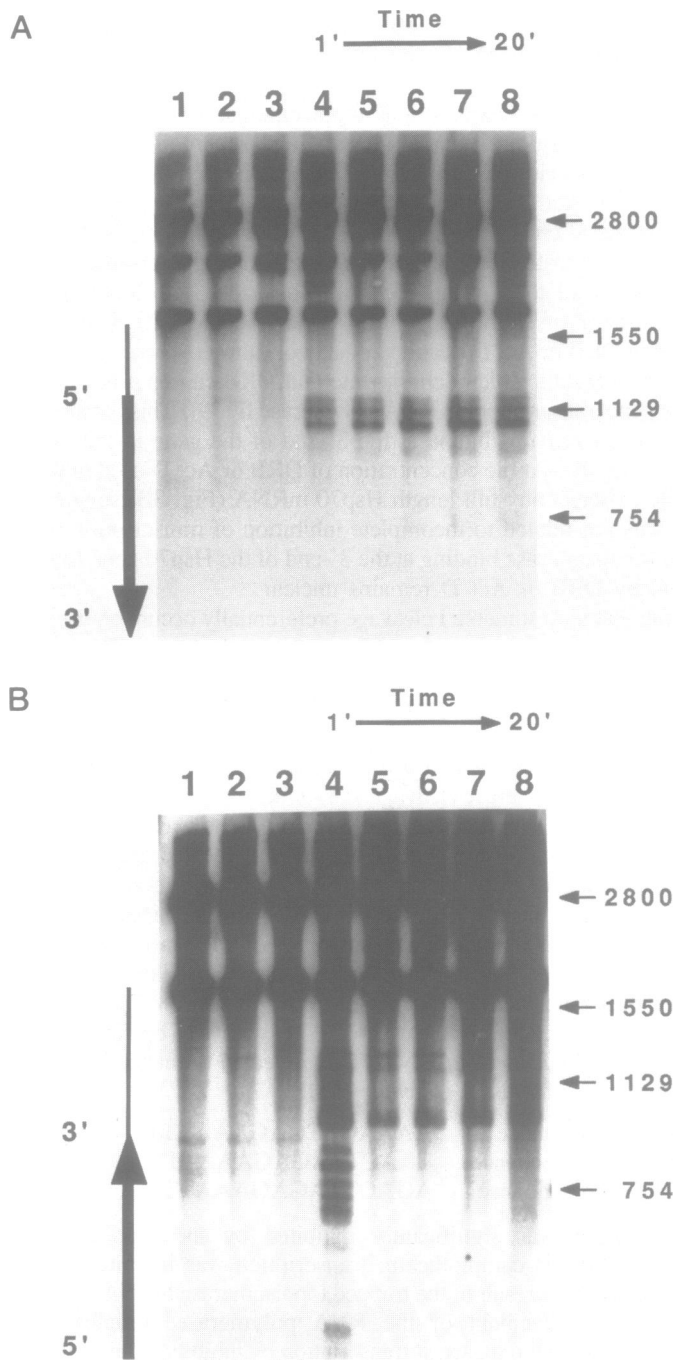
the moving transcription complex (8−10). Since topoisomerase I can remove both postitive and negative supercoils from DNA, this enzyme may facilitate transcription by relaxing supercoils which might otherwise accumulate and arrest the movement of the transcription complex. Such a role would explain why topoisomerase I binding is primarily localized within the transcribed region of active genes where supercoiling of the DNA would be greatest. In agreement with this model we find that inhibition of ongoing Hsp 70 RNA synthesis by DRB or Act D abolishes topoisomerase I cleavage except at the 5'-end and to a lesser extent the 3'-end of the gene (Fig. 5). The residual cleavage by topoisomerase I at the 5'-end may be related to synthesis of short prematurely terminated transcripts which form in the presence of these drugs (33,34). Consistent with this hypothesis, we have detected the presence of short Hsp70 transcripts (100 to 200 bases in length) which accumulate during DRB or Act D treatment (data not shown). These results are in agreement with recent *in vitro* results which demonstrate that DRB causes premature termination of adenovirus late gene transcription by RNA polymerase II (39). This, however cannot explain why topoisomerase I remains bound at the 3'-end of the gene in the presence of Act D or DRB (Fig. 4 and 5B). At the concentration of DRB or Act D used in these experiments we were unable to detect any full length Hsp70 mRNA (Fig. 3B) suggesting that binding in this region was not related to incomplete inhibition of transcription by this drug. The significance of topoisomerase binding at the 3'-end of the Hsp70 gene following inhibition of transcription by DRB or Act D remains unclear.

It is interesting that topoisomerse I cleavage preferentially occurs on the transcribed DNA strand of the Hsp70 gene (Fig. 3A). A similar result has been seen for the small Hsp genes in *Drosophila*, the rat tyrosine aminotransferase gene and the ribosomal genes in *Dictyostelium* (14,18,21). This assymmetry in binding may indicate that the transcribed strand is more readily accessible to topoisomerase I binding. Alternatively, the transcribed strand may contain structures which are good substrates for enzyme binding.

In addition to the topoisomerase cleavage sites which occurred throughout the transcribed region of the Hsp70 gene, a prominent topoisomerase I site was observed in the regulatory element flanking the 5'-end of the gene. This site was located approximately 150 to 200 bp upstream from the start of transcription (Fig. 2). Cleavage at this site was abolished by Act D or DRB (Fig. 3, lanes 4−6). Ness et al. (21) has identified several topoisomerase I sites between 130 to 300 nucleotides upstream of the start of transcription for the *Dictyostelium* rRNA genes. As is shown below, there is striking homology (12/16 bp) between one of these sites and a sequence at −180 in the *Drosophila* Hsp70 regulatory element (40). These sequences also share homology with a 16 bp repeated sequence found in the rRNAgenes of *Tetrahymena* DNA. This repeated sequence has been shown to be a strong site for topoisomerase I cleavage (41).

Drosophila sequence	AAAACTCGAGAAATTT
Dictyostelium sequence	TAACTTAGAGAAATTT
Tetrahymena sequence	AGACTTAGAGAAATTT

Hsp70 transcription was significantly inhibited by the topoisomerase I inhibitor camptothecin. At 100 μM camptothecin, transcription was inhibited greater than 95%. Inhibition may simply be a result of the trapped topoisomerase I-DNA complexes physically interfering with the movement of the RNA polymerase complex along the gene. Alternatively, camptothecin may arrest transcription by inhibiting topoisomerase I catalyzed relaxation of the DNA supercoils generated during transcription. This latter possibility


A
Time
1'
20'
1 2 3 4 5 6 7 8
2800
1550
1129
754
5'
3'
B
Time
1'
20'
1 2 3 4 5 6 7 8
2800
1550
1129
754
3'
5'

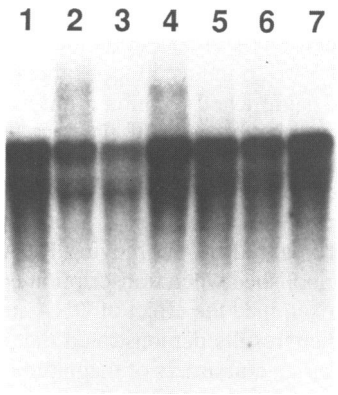


Figure 6. *Camptothecin inhibits Hsp70 RNA synthesis.* Cells preincubated for 5 min with either camptothecin (lanes B−D) or VM-26 (lanes E−G) were then heat-shocked for 15 min at 37°C. The RNA was then isolated and analyzed by Northern blotting as described in MATERIALS AND METHODS. Lanes A−D, 0, 2, 20 and 100 μM camptothecin respectively; Lanes E−G are 2, 20 and 100 μM VM-26, respectively. Lane H is control RNA from cells treated with 0.5% DMSO.

has been suggested by *in vitro* transcription studies in extracts from rat mammary adenocarcinoma cells (42). Transcription from a supercoiled ribosomal DNA template in these extracts was completely abolished by 150 μM camptothecin. This inhibition could be overcome if excess exogenous topoisomerase I was added to the reaction. In addition, if a linear ribosomal DNA template was used in the reaction, camptothecin had no affect on transcription suggesting that camptothecin interfered with transcription by inhibiting topoisomerase catalyzed changes in DNA topology rather than through the formation of topoisomerase I-DNA complexes which physically blocked the movement RNA polymerase through the gene. Camptothecin has also been shown to inhibit nuclear run-on transcription of ribosomal DNA in HeLa cells (19). Inhibition resulted in premature termination of the run-on transcripts near the 5′-end of the ribosomal gene suggesting that camptothecin interfered with the elongation step in RNA synthesis. However, these studies were unable to distinguish whether premature termination resulted from the inhibition of topoisomerase I catalyzed relaxation of DNA supercoils or from the physical presence of trapped topoisomerase I-DNA complexes on the ribosomal gene.

In contrast to camptothecin, the topoisomerase II inhibitor VM-26 had little effect on Hsp70 transcription. At 100 μM VM-26, Hsp70 RNA synthesis was inhibited less than 15% suggesting that this enzyme is not required for Hsp70 transcription. VM-26 has been

Figure 5. *Loss of topoisomerase I cleavage from transcriptionally active Hsp70 genes following DRB treatment.* Cells which had been heat shocked for 5 min at 37°C were treated with DRB (65 μM) to arrest transcription. Camptothecin was then added to 100 μM at 0, 3, 5, 10 and 20 min (lanes 4−8) after the addition of DRB and the cells were lysed 1 min later. The DNA was isolated and digested with BamHI and electrophoresed in duplicate 1.4% alkaline agarose gels as described in MATERIALS AND METHODS. The gels were then blotted onto nitrocellulose and subsequently probed with either nick-translated pMR2 (Panel A) or pMR4 (Panel B) DNA to map topoisomerase I sites in the 5′ or 3′ region of the Hsp70 gene respectively. Lanes: 1, 25°C control, no camptothecin or DRB; 2, 25°C in the presence of 100 μM camptothecin for 1 min; 3, 37°C control, no camptothecin or DRB; 4−8, time course of camptothecin addition (0, 3, 5, 10, and 20 min) after DRB treatment. (B) 3′ end of the Hsp70 gene. Molecular weight standards are denoted at the right and are given in bases.

shown to stabilize a cleavable complex between topoisomerase II and DNA which results in protein linked double-stranded DNA breaks upon the addition of a protein denaturant (36). The physical presence of VM-26 induced topoisomerase II-DNA complexes on the Hsp70 gene do not appear to significantly inhibit transcription of this gene by RNA polymerase. However, this may be because of the fact that there are relatively few topoisomerase II cleavage sites on the Hsp70 gene and these sites are localized to the 5′ and 3′ ends of the gene (15).

The binding of topoisomerase I to the Hsp70 gene is most likely a response to perturbations in DNA structure caused by the moving RNA polymerase complex since binding can almost be entirely abolished when transcription is inhibited by Act D or DRB. Camilloni et al. have recently examined the effect of DNA supercoiling on topoisomerase I cleavage *in vitro* (43,44). Their results demonstrated that topoisomerase I cleavage of a supercoiled DNA template was several orders of magnitude greater than that of a relaxed DNA template. This would suggest that cleavage of transcriptionally active Hsp70 DNA by topoisomerase I would most likely occur near the RNA polymerase complex, a place where DNA supercoiling would be greatest. Our DRB and Act D results support this idea. In the presence of these drugs, topoisomerase cleavage was primarily confined to the first 200 bases at the 5′-end of the transcribed region. This closely correlates with the size of premature Hsp70 transcripts which are formed in heat-induced cells treated with either Act D and DRB indicating that topoisomerase I is acting on DNA that is near the transcription complex. Although topoisomerase I may be acting in close proximity to RNA polymerase, it is probably not an integral component of the polymerase complex. Photo cross-linking experiments have been done to measure the relative binding of topoisomerase I and RNA polymerase II to transcriptionally active Hsp70 and copia sequences in *Drosophila* DNA (13). The ratio of topoisomerase I to RNA polymerase molecules that were photo cross-linked to transcriptionally active Hsp70 sequences was significantly different from the ratio on the copia gene suggesting that topoisomerase I was not an integral part of the transcription complex.

ACKNOWLEDGEMENTS

The authors wish to thank David Kroll, Chris Borgert, and Jeff Lawrence for helpful discussions during the course of these studies. This work was supported by Public Health Service grant GM38859 from the National Institutes of Health.

ABBREVIATIONS

Hsp, heat shock protein; Act D, Actinomycin D; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; SDS, sodium dodecyl sulfate; DMSO, dimethylsulfoxide; 5× SSPE = 0.9 M NaCl, 50 mM $NaPO_4$, pH7.7, 5 mM EDTA.

*To whom correspondence should be addressed

REFERENCES

1. Liu, L.F. (1983) Crit. Rev. Biochem **15**, 1–24.
2. Wang, J. (1985) Ann. Rev. Biochem. **54**, 665–699.
3. Vosberg, P.-H. (1985) Curr. Top. Micrbiol. Immunol. **114**, 19–102.
4. Rudd, K.E. and Menzel, R. (1987) Proc. Natl. Acad. Sci. USA **84**, 517–521.
5. Richardson, S.M.H., Higgins, C.F., Lilley, D.M.J. (1988) EMBO J. **7**, 1863–1869.
6. Brill, S. J., Dinardo, S., Voelkel-Meiman, K., Sternglanz, R. (1987) Nature **326**, 414–416.

7. Egyhazi, E. and Durban, E. (1987) Mol. Cell. Biol. **7**, 4308–4316.
8. Wu, H.-Y., Shyy, S., Wang, J.C., and Liu, L.F. (1988) Cell **53**, 433–440.
9. Giaever, G.N. and Wang, J.C. (1988) Cell **55**, 849–856.
10. Tsao, Y.-P., Wu, H.-Y., and Liu, L.F. (1989) Cell **56**, 111–118.
11. Pruss, G.J. and Drlica, K. (1989) Cell **56**, 521–523.
12. Brill, S J., Sternglanz, R. (1988) Cell **54**, 403–411.
13. Gilmour, D. S., Pflugfelder, G., Wang, J. C., Lis, J. T. (1986) Cell **44**, 401–407.
14. Gilmour, D. S., Elgin, S. C. R. (1987) Mol. Cell. Biol. **7**, 141–148.
15. Rowe, T.C., Wang, J.C., and Liu, L.F. (1986) Mol. Cell. Biol. **6**, 985–992.
16. Linquist, S. (1986) Ann. Rev. Biochem **55**, 1151–1191.
17. Hsiang, Y-H., Hertzberg, R., Hecht, S., Liu, L. F. (1985) J. Biol. Chem. **260**, 14873–14878.
18. Stewart, A.F., and Schutz, G. (1987) Cell **50**, 1109–1117.
19. Zhang, H., Wang, J. C., Liu, L. F. (1988) Proc. Natl. Acad. Sci. USA **85**, 1060–1064.
20. Culotta, V., Sollner-Webb, B. (1988) Cell **52**, 585–597.
21. Ness, P.J., Koller, T., and Thoma, F. (1988) J. Mol. Biol. **200**, 127–139.
22. Fraser, N.W., Sengal, P.B., Darnell, J.E. (1978) Nature **272**, 590–593.
23. Sobell (1985) Proc. Natl. Acad. Sci. USA **82**, 5328–5331.
24. Corces, V., Pellicer, A., Axel, R., and Meselson, M. (1978) Proc. Natl. Acad. Sci. USA **78**, 7038–7042.
25. Schneider, I. and Blumenthal, A.B. (1978). In *The Genetics and Biology of Drosophila* (eds. M. Ashburner and T.R.F. Wright) Vol. **2a**, 266–315, Academic Press, N.Y.
26. Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159.
27. Maniatis, T., Fritsch, E., and Sambrook, J. (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
28. Melton, D.A., Krieg, P.A., Rebagianti, M.R., Maniatis, T., Zinn, K., Green, M.R. (1984) Nucl. Acids Res. **12**, 7035–7056.
29. Livak, K.J., Freund, R., Schweber, M., Wensink, P.C., and Meselson, M. (1978) Proc. Natl. Acad. Sci. USA **75**, 5613–5617.
30. Mirault, M.-E., Goldschmidt-Clermont, M., Artavanis-Tsakonas, S., and Schedl, P. (1979). Proc. Natl. Acad. Sci. USA **76**, 5254–5258.
31. Wu, C. (1980) Nature (London) **286**, 854–859.
32. Trask, D.K. and Muller, M.T. (1988) Proc. Natl. Acad. Sci. USA **85**, 1417–1421.
33. Fraser, N.W., Sehgal, P.B., and Darnell, J.E. (1979) Proc. Natl. Acad. Sci. USA **76**, 2571.
34. Tamm, I., and Kikuchi, T. (1979) Proc. Natl. Acad. Sci. USA **76**, 5750–5754.
35. Horwitz, S.B., Chang, C.-K., and Grollman, A.P. (1971) Molec. Pharm. **7**, 632–644.
36. Chen, G.L., Yang, L., Rowe, T.C., Halligan, B.D., Tewey, K.M., and Liu, L.F. (1984) J. Biol. Chem. **259**, 13560–13566.
37. Fleischmann, G., Pjlugfelder, G., Steiner, E.K., Javaherian, K., Howard, G.C., Wang, J.C., and Elgin, S.C.R. (1984) Proc. Natl. Acad. Sci. USA **81**, 6958–6962.
38. Weisbrod, S. (1982) Nature **297**, 289–295).
39. Chodish, L.A., Fire, A., Samuels, M., and Sharp, P.A. (1989) J. Biol. Chem. **264**, 2250–2257.
40. Mason, P.J., Torok, I., Kiss, I., Karch, F., and Udvardy, A. (1982) J. Mol. Biol. **156**, 21–35.
41. Bonven, B.J., Gocke, E., Westergaard, O. (1985) Cell **41**, 541–551.
42. Garg, L. C., DiAngelo, S., Jacob, S. T. (1987) Proc. Natl. Acad. Sci. USA **84**, 3185–3188.
43. Camilloni, G., Di Martino, E., Caserta, M., and Di Mauro, E. (1988) Nucleic Acids Res. **16**, 7071–7085.
44. Camilloni, G., Di Martino, E., Di Mauro, E., and Caserta, M. (1989) Proc. Natl. Acad. Sci. USA **86**, 3080–3084.

This article, submitted on disc, has been automatically converted into this typeset format by the publisher.