de Leeuw, F. A. A. M., & Altona, C. (1982) *J. Chem. Soc., Perkin Trans. 2*, 375–384.
de Leeuw, F. A. A. M., & Altona, C. (1983) *QCPE Bull. 3*, 69–70.
Lewin, B. (1983) *Genes*, Wiley, New York.
Remin, M., & Shugar, D. (1972) *Biochem. Biophys. Res. Commun. 48*, 636–642.
Ross, P., Weinhouse, H., Aloni, Y., Michaeli, D., Weinberger-Ohana, P., Mayer, R., Braun, S., de Vroom, E., van der Marel, G. A., van Boom, J. H., & Benziman, M. (1987) *Nature (London) 325*, 279–281.
Still, W. C. (1986) MacroModel, Columbia University, New York.
Tabak, H. F., van der Horst, G., Kamps, A. M. J. E., & Arnberg, A. C. (1987) *Cell (Cambridge, Mass.) 48*, 101–110.
de Vroom, E., van der Marel, G. A., & van Boom, J. H. (1988) *Nucleic Acids Res.* (submitted for publication).
Watson, J. D., Hopkins, N. H., Roberts, J. W., Steitz, J. A., & Weiner, A. M. (1987) *Molecular Biology of the Gene*, 4th ed., Benjamin/Cummings, Menlo Park, CA.
Weiner, S. J., Kollman, P. A., Case, D. A., Singh, U. C., Ghio, C., Alagona, G., Profeta, S., & Weiner, P. (1984) *J. Am. Chem. Soc. 106*, 765–784.

Single-Strand DNA Cleavages by Eukaryotic Topoisomerase II†

M. T. Muller,*,‡ J. R. Spitzner,‡ J. A. DiDonato,‡ V. B. Mehta,‡ Kimiko Tsutsui,§ and Ken Tsutsui‖
Department of Molecular Genetics, The Ohio State University, Columbus, Ohio 43210, and Department of Clinical Neurochemistry, Institute for Neurobiology, and Department of Biochemistry, Cancer Institute, Okayama University Medical School, Okayama, 700 Japan
Received January 12, 1988; Revised Manuscript Received May 27, 1988

ABSTRACT: A new purification method for eukaryotic type II DNA topoisomerase (EC 5.99.1.3) is described, and the avian enzyme has been purified and characterized. An analysis of the cleavage reaction has revealed that topoisomerase II can be trapped as a DNA–enzyme covalent complex containing DNA with double-stranded and single-stranded breaks. The data indicate that DNA cleavage by topoisomerase II proceeds by two asymmetric single-stranded cleavage and resealing steps on opposite strands (separated by 4 bp) with independent probabilities of being trapped upon addition of a protein denaturant. Single-strand cleavages were directly demonstrated at both strong and weak topoisomerase II sites. Thus, a match to the vertebrate topoisomerase II consensus sequence

	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	1	2	3	4	5	6	7	8
5'	A/G	N	C/T	N	N	C	N	N	G	T/C	N	G	G/T	T	N	T/C	N	T/C

(N is any base, and cleavage occurs between −1 and +1) [Spitzner, J. R., & Muller, M. T. (1988) *Nucleic Acids Res. 16*, 5533–5556)] does not predict whether a cleavage site will be single stranded or double stranded; however, sites cleaved by topoisomerase II that contain two conserved consensus bases (G residue at +2 and T at +4) generally yield double-strand cleavage whereas recognition sites lacking these two consensus elements yield single-strand cleavages. Finally, single-strand cleavages with topoisomerase II do not appear to be an artifact caused by damaged enzyme molecules since topoisomerase II in freshly prepared, crude extracts also shows the property of single-strand cleavages.

A single theme unifies the action of topoisomerases: These enzymes can adjust the topological state of DNA by breaking and resealing DNA strands, allowing for alterations in the linking number [for reviews, see Vosberg (1985) and Wang (1985)]. Two major categories of topoisomerases are recognized. The type I enzymes alter DNA linking number by a transient break in one strand through which the opposing (intact) strand can pass. Type II topoisomerases transiently break both strands which produces a double-strand DNA–protein gate through which an intact section of the duplex can pass.

Eukaryotic type II topoisomerases, originally identified in *Drosophila melanogaster* embryos (Hsieh & Brutlag, 1980) and *Xenopus laevis* oocytes (Baldi et al., 1980), have subsequently been purified from a number of different eukaryotic species (Miller et al., 1981; Goto & Wang, 1982; Shelton et al., 1983; Benedetti et al., 1983; Duoc-Rasy et al., 1986), and the availability of specific antibodies (Earnshaw & Heck, 1985; Berrios et al., 1985; Spitzner and Muller, unpublished results) and mutants (Thrash et al., 1984; Uemura & Yanagida, 1984; Holm et al., 1985) has revealed several possible functions of these enzymes. Notably, topoisomerase II is essential for segregation of daughter chromatids after DNA replication (Uemura & Yanagida, 1984; DiNardo et al., 1984; Holm et al., 1985) and is enriched in the nuclear matrix–pore complex (Berrios et al., 1985). These observations imply a tight coupling between topoisomerase II and cell cycle activity (Duguet et al., 1983; Uemura & Yanagida, 1986). Less is known about a direct relationship between topoisomerase II activity and cell differentiation although in brain tissue topoisomerase II activity has been shown to remain high in terminally differentiated cells (Tsutsui et al., 1986). In avian erythrocytes, less topoisomerase II activity is present in terminally differentiated (mature) erythrocytes (Spitzner and Muller, unpublished data), suggesting that generalizations may not hold for all tissues.

† The work was supported by Public Health Service Grant GM31640 from the National Institutes of Health and by Grant 1-1031 from the March of Dimes Foundation.
‡ The Ohio State University.
§ Institute for Neuobiology, Okayama University Medical School.
‖ Cancer Institute, Okayama University Medical School.

0006-2960/88/0427-8369$01.50/0

The avian system provides an opportunity to evaluate the relationship between topoisomerase II, active gene structures, and cell differentiation since certain cell lineages, like erythrocytes, are amenable to experimental manipulation. Furthermore, the chicken enzyme was found to be associated with the metaphase chromosome scaffold by use of immunological methods (Earnshaw & Heck, 1985); thus, it was of interest to purify the enzyme and characterize its reaction mechanism with DNA. In particular, we addressed an issue that is fundamental in the distinction of type I and II topoisomerases, that is, the formation of single-strand nicks by a type II topoisomerase. Two previous observations prompted us to analyze single-strand cleavages with purified topoisomerase II: first, the fact that topoisomerase II can complement topoisomerase I deletions in yeast (Uemura & Yanagida, 1984; Brill et al., 1987), and second, the finding that drugs which stabilize the covalent topoisomerase/DNA intermediate (Nelson et al., 1984; Tewey et al., 1984) produce double- and single-stranded breaks in vivo (Ross et al., 1979; Zwelling et al., 1981). In the present study, we report a rapid purification of topoisomerase II and provide evidence that topoisomerase II, like type I enzymes, can generate nicked, open circular DNA intermediates from circular DNA when the reaction is interrupted with a detergent.

Experimental Procedures

Enzymes and DNA. Supercoiled pBR322 was prepared as described (Maniatis et al., 1982). Kinetoplast DNA (kDNA)[1] was purified from *Crithidia fasciculata* (provided by P. Englund) according to methods originally described by Saucier et al. (1981) with modifications described by Hajduk et al. (1984). Following ethanol precipitation, kDNA was resuspended in TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) to a final concentration of 0.25 mg/mL. End-labeled DNA fragments were prepared by either a fill-in reaction with *Escherichia coli* DNA polymerase I (Klenow) for 3′ ends or by polynucleotide kinase for 5′ ends (Maniatis et al., 1982; Maxam & Gilbert, 1977). Sequencing reactions were carried out as described (Maxam & Gilbert, 1977).

Enzyme Assays. Topoisomerase I was assayed as described previously (Trask & Muller, 1983) with supercoiled pBR322 DNA as substrate. Type II topoisomerase was assayed by decatenation of kDNA (Marini et al., 1980) and monitoring the appearance of a monomer DNA of 2.5 kilobases (kb). Reactions contained 0.25 μg of kDNA (final volume of 20–30 μL), 50 mM Tris-HCl, pH 7.9, 120 mM KCl, 10 mM $MgCl_2$, 30 μg of BSA/mL, and 0.5 mM each of dithiothreitol, EDTA, and ATP (topoisomerase II reaction buffer). Reactions were incubated for 5–30 min at 30 °C, terminated with SDS (0.5% final), and digested with proteinase K (50 μg/mL) for 15 min at 50 °C followed by addition of bromophenol blue (0.005%) and glycerol (4%). The products were analyzed on 1% agarose gels containing 0.5 μg of ethidium bromide/mL to resolve nicked and closed circular DNAs. Electrophoresis was performed at 5–10 V/cm which allowed rapid (10 min) resolution of catenated networks from the 2.5-kb minicircles as monitored with a hand-held UV light. One unit of topoisomerase II is defined as the amount of enzyme required to completely decatenate 0.25 μg of kDNA substrate in a 15-min reaction of 30 °C. Covalent complexes between topoisomerases and DNA were analyzed by the SDS-K^+ precipitation method described previously (Trask et al., 1984).

Analysis of DNA Cleavages. Reactions were carried out in a final volume of 20 μL in a cleavage buffer containing the following final concentration of solutes: 20 mM Tris-HCl, pH 8.0, 3 mM mercaptoethanol, 4 mM $MgCl_2$, 100 mM NaCl, 3 mM ATP, and 0.1 mg of BSA/mL. Where indicated, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) was added to the reaction just prior to addition of the topoisomerase II. The final concentrations of drugs are indicated in the experiments. Reactions were incubated at 30 °C for 20 min and stopped by the addition of 2 volumes of 1.5% SDS followed by digestion with proteinase K as above. After addition of 0.3 volume of 4.5 M ammonium acetate, the DNA was ethanol precipitated, and following a wash with 70% ethanol, the pellet was suspended in loading buffer for sequence analysis (Maxam & Gilbert, 1977).

Purification of Topoisomerase II. All steps were carried out at 0–4 °C. Fresh chicken blood was collected (mixed with heparin) from a local slaughterhouse, and 4 L of blood was concentrated to 2 L by centrifugation (13000*g*, 10 min) and then diluted with an equal volume of buffer A [10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 5 mM $MgCl_2$, 0.5 mM phenylmethanesulfonyl fluoride (PMSF)]. The centrifugation step was repeated, and the cell pellets were resuspended in 2 L of buffer A. The suspension was then frozen (−70 °C) for 12–24 h. The cells can be kept in this state indefinitely. This freezing step serves to lyse the cells and release nuclei. The cell suspensions were then thawed at 37 °C with gentle stirring with a glass rod, followed by centrifugation (13000*g* 10 min). The resulting nuclei were resuspended in one pellet volume of buffer A, and the centrifugation step was repeated. The washing steps with buffer were continued until most of the hemoglobin was removed, usually 4 times. The final pellet of nuclei was resuspended to a final volume of 800 mL in buffer B (50 mM Tris-HCl, pH 7.6, 50 mM sodium bisulfite, 30 mM 2-mercaptoethanol, 1 mM PMSF). Solid NaCl was added to a final concentration of 0.3 M, and the suspension was stirred for 60 min followed by the addition of 10% poly(ethylenimine) to give a final concentration of 0.9%. The lysate was stirred briskly for 30 min and centrifuged (13000*g*, 20 min), and the supernatant (650 mL) was retained. Solid ammonium sulfate (118.3 g) was slowly added, and after being stirred for 60 min, the precipitate was removed by centrifugation (13000*g*, 20 min), and an additional 61.6 g of ammonium sulfate was added to the supernatant. The stirring and centrifugation steps were repeated, and the precipitated protein was recovered by resuspension in 100 mL of buffer C [0.1 M potassium phosphate (KP_i), pH 7.1, 10% glycerol, 25 mM 2-mercaptoethanol, 10 mM sodium bisulfite, 0.5 mM PMSF]. This fraction was rich in topoisomerase II.

A Bio-Rex 70 column (2.5 × 30 cm), preequilibrated in buffer C containing 0.2 M KP_i, was loaded with 80 mL of the above fraction and then washed with the equilibration buffer until all residual hemoglobin was removed (about 3 column volumes). The column was developed with a 600-mL linear gradient of buffer C from 0.2 to 0.7 M KP_i, at a flow rate of 60 mL/h. Gradient fractions (9 mL) were assayed by decatenation assays and by relaxation of plasmid DNA (the latter reactions lacked ATP and $MgCl_2$ to detect only topoisomerase I). The peak fractions of topoisomerase II activity, around 0.4–0.45 M KP_i, were pooled and loaded directly onto a phenyl-Sepharose column (1 × 6 cm) previously equilibrated with buffer D (20% ethylene glycol, 25 mM KP_i, pH 7.1, 25

[1] Abbreviations: kDNA, kinetoplast DNA; *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; SDS, sodium dodecyl sulfate; SDS-K+, precipitate formed upon addition of KCl to SDS and used to precipitate protein and DNA–protein complexes; KP_i, potassium phosphate buffer; PMSF, phenylmethanesulfonyl fluoride; IE3, immediate early gene 3; HSV-1, herpes simplex virus type 1; topo, topoisomerase.

Table I: Topoisomerase II Purification Summary

step	protein concn (mg)	activity (units)	sp act. (units/mg)	yield (%)
0.3 M NaCl extract	13000	4.4×10^5	34	100
polymin-P precipn	3800		*a*	
$(NH_2)_2SO_4$ precipn	80	3.2×10^5	4×10^3	73
Bio-Rex pool	5.4	1.9×10^5	3.5×10^4	43
phenyl-Sepharose	1.2	5×10^4	4.2×10^4	11
hydroxyapatite	0.2	3.7×10^4	1.7×10^5	8.4

[a] Due to interference with the polycation at this step, activity determinations were not possible.

mM 2-mercaptoethanol, 10 mM sodium bisulfite, 0.5 mM PMSF). The flow rate was 90 mL/h. The column was washed with 40 mL of buffer C and the activity eluted with buffer D containing 60% ethylene glycol. A gradient was not required at this step. In some cases, in order to concentrate the activity and attain a slight increase in specific activity, the phenyl-Sepharose pool was loaded directly onto a hydroxyapatite column (0.8 × 3 cm) equilibrated in buffer C. A series of batch elutions in buffer C (3 column volumes each of 0.2, 0.3, and 0.4 M KP_i) were performed. The 0.4 M fractions contained topoisomerase II, free of ethylene glycol and in concentrated form. The higher salt in this fraction helped to stabilize the activity when stored at 4 °C (up to 6 weeks); however, the activity decreased variably with repeated freezing and thawing. The enzyme was stored in small aliquots at −80 °C which were not refrozen after thawing. More recently, the phenyl-Sepharose pool was found to be quite stable when stored unfrozen (in glycerol or ethylene glycol) at −20 °C.

Results

Characterization of Chicken Topoisomerase II. Chicken erythrocytes are a rich source of type I topoisomerase (Trask & Muller, 1983). Decatenation assays employing kDNA as substrate revealed that nuclear extracts also contained topoisomerase II. While topoisomerase I was efficiently extracted from nuclei with 0.4 M NaCl as described previously (Trask & Muller, 1983), we found that 0.3 M NaCl extracted similar amounts of topoisomerase II and much less histone H1, which has been shown to promote topoisomerase II mediated catenation of DNA (Hsieh & Brutlag, 1980; Shelton et al., 1983). Since our assay measures the reverse reaction (decatenation), elimination of H1 was important. We also found that the addition of relatively high levels of 2-mercaptoethanol (0.15 M) extracted larger amounts of topoisomerase II presumably by dissociating it from matrix/scaffold structures (Gasser et al., 1986); however, purity was not as high in the final fraction with the purification described here. Therefore, extracting topoisomerase II along with nuclear matrix/scaffold associated proteins resulted in contaminating proteins that we could not remove in the two-column purification procedure. Prior to ammonium sulfate fractionation, contaminating nucleic acids and acidic proteins were precipitated with poly(ethylenimine) (Jendrisak & Burgess, 1975). Two ammonium sulfate cuts removed some contaminating topoisomerase I and resulted in a significant increase in specific activity of topoisomerase II (see Table I). The extract was first chromatographed over Bio-Rex 70, which resulted in partial separation of topoisomerase I. In addition, the bulk of the protein did not bind the column, and a significant purification was achieved (data not shown). The Bio-Rex 70 pool was loaded directly onto phenyl-Sepharose without an intervening dialysis (or dilution) step. The residual topoisomerase I elutes in the break-through fractions, and with subsequent washes of the column (20% ethylene glycol, 25 mM KP_i) most proteins are

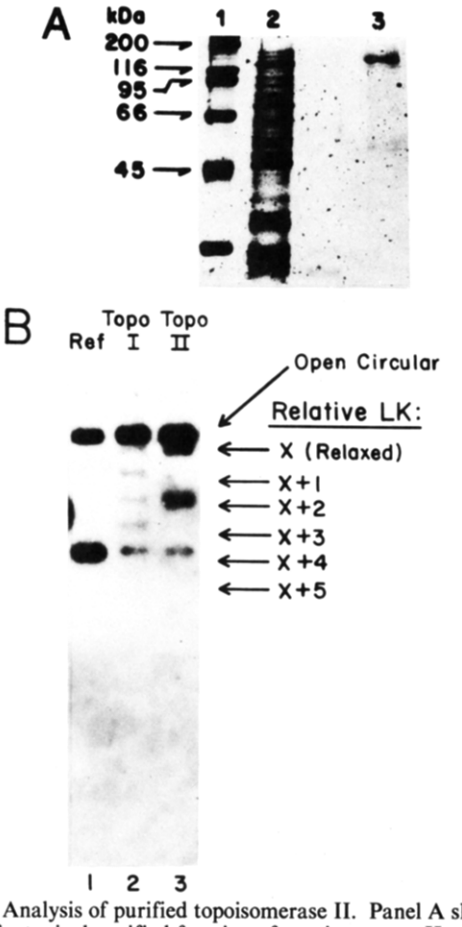


FIGURE 1: Analysis of purified topoisomerase II. Panel A shows an analysis of a typical purified fraction of topoisomerase II on a 12% SDS polyacrylamide gel. Markers are shown in lane 1 and their molecular weights given on the left. Lane 2 contains approximately 100 μg of the ammonium sulfate fraction taken just prior to loading the first ion-exchange column. Lane 3 contains 1 μg of purified topoisomerase II fraction from a phenyl-Sepharose column eluted with 60% ethylene glycol. The gel was stained with Coomassie Blue. Panel B shows an analysis of alteration of DNA linking number by purified avian topoisomerase II. A unique topoisomer of pBR322 (25 ng) was isolated as described previously (Trask & Muller, 1983) and incubated with 2 units each of purified topoisomerase I (lane 2) or purified avian topoisomerase II (lane 3). The topoisomer alone is shown in lane 1. Reaction products were separated by electrophoresis in a 1% agarose gel and the bands detected by Southern blotting. Relative linking numbers are given on the right side of (B).

desorbed except topoisomerase II, which elutes with 60% ethylene glycol. A summary of the purification is given in Table I. Starting with the equivalent of 1 L of whole blood, about 200 μg of enzyme was recovered with an 8% yield. In the purification scheme shown in Table I, we included an extra step to concentrate the activity; however, this is not routinely necessary. The polyacrylamide gel (Figure 1A) shows that the phenyl-Sepharose fraction contains a prominent band of 155–160 kDa, which is in the expected size range. This fraction was also analyzed on Western blots with an antibody to a major chicken scaffold protein which has been shown to be chicken topoisomerase II (Earnshaw & Heck, 1985). This antibody reacted with the 155-kDa polypeptide (data not shown), as does a monoclonal antibody we raised against chicken topoisomerase II (Spitzner and Muller, unpublished results).

To demonstrate that the final fraction is a type II topoisomerase and to rule out contamination by topoisomerase I, we analyzed the alterations in DNA linking number (Figure 1B). A unique topoisomer was prepared and shown to be relaxed in steps of one by topoisomerase I (lane 2) and in steps

Table II: Conditions for Topoisomerase II Binding to DNA[a]

conditions	% of input DNA in SDS-K+ precipitate: −AMSA	+AMSA (concn indicated)
experiment 1		
complete	15	51 (1 μg/mL)
complete	15	47 (10 μg/mL)
complete	15	36 (100 μg/mL)
experiment 2		
complete	24	62 (1 μg/mL)
−ATP	4	31 (1 μg/mL)
$-MgCl_2$	3	5 (1 μg/mL)
$-ATP/-MgCl_2$	3	5 (1 μg/mL)
$-ATP/-MgCl_2/+CaCl_2$	6	12.5 (1 μg/mL)

[a] Reactions were performed with 2.5 ng of DNA (5′ end-labeled DNA fragment described in Figure 2). Experiments 1 and 2 were carried out with different preparations of topoisomerase II.

of two by topoisomerase II (lane 3). Lengthy exposures of the blot in Figure 1B revealed that there was essentially no contaminating topoisomerase I in the phenyl-Sepharose pool. Furthermore, it was not possible to detect activity when a 10-fold excess of purified topoisomerase II was incubated for 2 h with supercoiled DNA in the presence of $MgCl_2$ but without ATP. These conditions should reveal relatively low levels of topoisomerase I contamination. Finally, we have used this purification procedure with different tissues and cell types, notably with human cells (HeLa and placenta) and chicken MSB-1 cells (Spitzner & Muller, 1988).

Covalent Complexes of Topoisomerase II and DNA. We previously reported that a topoisomerase II/DNA intermediate is readily detected by incubating labeled DNA with purified topoisomerase II, trapping the complexes with SDS, and precipitating the protein–DNA complexes with KCl (Trask et al., 1984). We refer to the procedure as the SDS-K+ precipitation assay. Usually, a small fraction of the input DNA (<20%) can be driven into covalent complexes; however, this fraction can be increased by treatment with *m*-AMSA. This drug stabilizes the covalent interaction between topoisomerase II and DNA (Nelson et al., 1984; Tewey et al., 1984) and elevates the SDS-K+ precipitation of DNA (Table II, experiment 1). The SDS-K+ signal is stimulated from 2- to 8-fold with *m*-AMSA depending on the stoichiometric ratios of enzyme and DNA and reaction conditions (see experiment 2). Also, Table II shows that the concentration of *m*-AMSA is not critical over the range tested. The ability of topoisomerase II to form a covalent (SDS-K+ precipitable) complex with DNA required Mg^{2+} (Trask et al., 1984; Sander & Hsieh, 1983; Udvardy et al., 1986), and ATP stimulated the SDS-K+ precipitation further (experiment 2) (Udvardy et al., 1986).

The linkage between DNA and topoisomerase II in the cleavage intermediate was deduced by use of differentially end-labeled DNA fragments and the SDS-K+ precipitation assay (Trask et al., 1984). Chicken topoisomerase II cleaves DNA and forms a 5′-phosphoryl linkage at the site of the break and generates a 3′-hydroxyl at the contiguous site (data not shown) as demonstrated with other eukaryotic type II enzymes (Sander & Hsieh, 1983; Liu et al., 1983).

Analysis of Cleavage at the DNA Sequence Level. The antitumor drugs VM-26 and *m*-AMSA inhibit the decatenation activity of our purified topoisomerase II (data not shown), and we used the drugs to stimulate cleavages of DNA fragments followed by DNA sequencing to analyze cleavage sites. A strong cleavage site was identified in the *lac* Z gene of pUC 19 [*Pvu*II–*Eco*RI fragment from nucleotide 306 to nucleotide

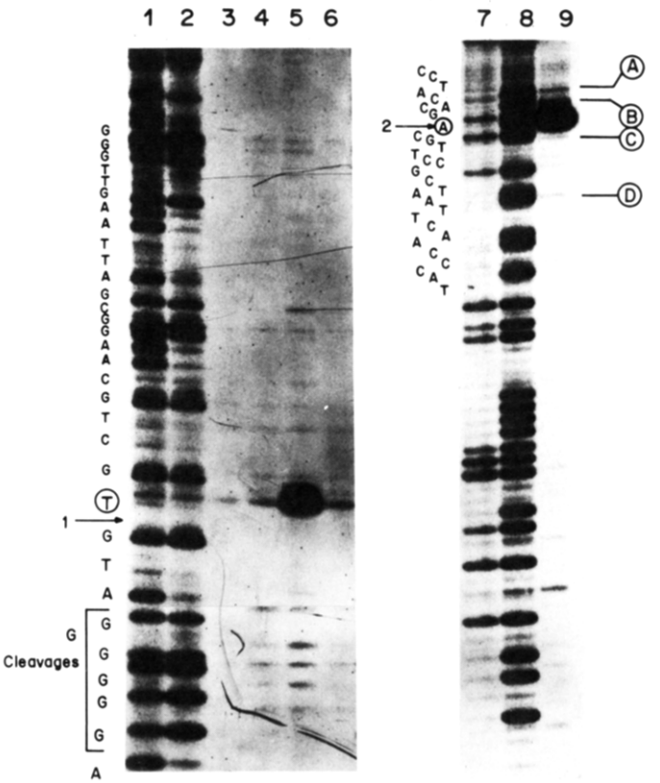


FIGURE 2: Topoisomerase II cleavages in *lac* Z gene region of pUC 19. Singly 5′ end-labeled fragments were prepared from pUC 19. Lanes 1–6 contained the 90-bp *Pvu*II–*Eco*RI fragment labeled at the *Pvu*II site, and lanes 7–9 contained the same fragment labeled on the opposite strand at the *Eco*RI site. The *Pvu*II and *Eco*RI sites correspond to positions 306 and 396, respectively, according to the sequence published by Yanisch-Perron et al. (1985). Cleavage reactions contained 6×10^4 dpm (ca. 8 ng) of each fragment and 4 units of purified topoisomerase II. The cleavage products were analyzed on a 20% sequencing gel. Lanes 2 and 7, G cleavage ladders; lanes 1 and 8, G+A ladders; lane 3, fragment untreated with topoisomerase II; lane 4, cleavage reaction without drugs; lanes 5 and 9, reactions containing 10 μg of *m*-AMSA/mL; lane 6, topoisomerase II reaction containing 25 μg of VM-26/mL. The major cleavage sites (marked with numbers, 1 for top strand or 2 for bottom strand) were oriented around the nucleotide circled in the sequence ladder on the left of the figure. Minor cleavages are indicated by letters, including the G cleavages which correspond to lanes 4 and 5. Additional cleavages below site D in lane 9 were not investigated.

396; see Yanisch-Perron et al. (1985)]. We compared cleavages at this site in the presence or absence of either VM-26 or *m*-AMSA, and by differentially labeling the 5′ ends, cleavage on each strand was evaluated (Figure 2). In the absence of the inhibitors, cleavage sites were detected on opposing strands at positions marked 1 and 2. Addition of *m*-AMSA stimulated the cleavage at these sites and revealed additional sites of weaker intensity. The cleavage reaction was also stimulated by VM-26 (a nonintercalator); however, VM-26 did not stimulate as effectively as *m*-AMSA, nor were additional sites revealed by VM-26. In general, we have found that cleavages at strong sites in the absence of drugs were stimulated by the drugs, although not all sites are stimulated equally and some cleavages are not stimulated at all (Spitzner & Muller, 1988). The reverse reaction was not observed; viz, the presence of the drugs did not silence sites that were evident in the absence of drugs. The drugs appear to divulge cleavages at a number of relatively weak sites that are barely detectable (or require long exposure times for detection) without *m*-AMSA. In otherwords, the majority of drug-stimulated cleavages are sites observed in the absence of drugs (Spitzner & Muller, 1988).

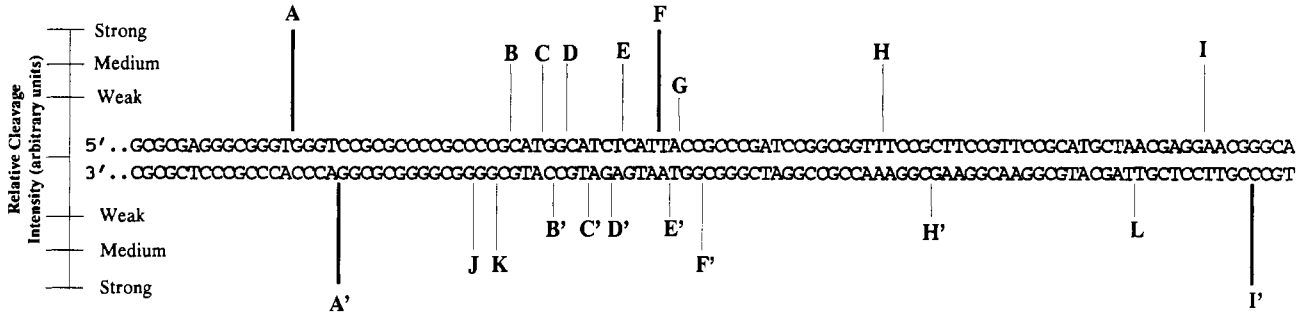
Table III: Summary of Cleavage Data[a]

Experiment 1 (from Figure 2): Cleavages in pUC 19 *Pvu*II–*Eco*RI

Experiment 2 (from Figure 3): Cleavages in HSV-1 IE Gene 3 *Eco*RI–*Nco*I

cleavage sites	homology to topo II consensus sequence[b] % match (top strand)	% match (bottom strand)	symmetry between sites	cleavage sites	homology to topo II consensus sequence[b] % match (top strand)	% match (bottom strand)	symmetry between sites
1 and 2	40	70	good	Y′ and Y	60	60	poor
A	70	40	good	II′ and II	60	70	good
B	40	70	poor	X	(60)	40	none
C	50	50	poor	I′ and I	80	40	good
D	(70)	50	none				

Experiment 3 (Data Not Shown): Cleavages in HSV-1 IE Gene 3 Enhancer *Nco*I–*Ava*I[c]



cleavage sites	homology to topo II consensus sequence[b] % match (top strand)	% match (bottom strand)	symmetry between sites	cleavage sites	homology to topo II consensus sequence[b] % match (top strand)	% match (bottom strand)	symmetry between sites
A and A′	90	40	good	G	40	(50)	none
B and B′	50	50	partial	H and H′	50	40	partial
C and C′	60	60	partial	I and I′	40	90	partial
D and D′	60	50	partial	J	(70)	40	none
E and E′	60	60	partial	K	(30)	70	none
F and F′	60	60	poor	L	(50)	70	none

[a] The data come from sequencing gels for top and bottom strands. In experiment 2, the numbers correspond to the position of the sequence with respect to the start site of transcription of IE gene 3 of HSV-1 (McGeoch et al., 1986). [b] Homology to the topo II consensus sequence was determined for each cleavage site in experiments 1 and 2 and the percent match (to non-N positions only) to top and bottom strands is shown. In cases where we could not assign an opposing strand cleavage (e.g., site J or K, experiment 3), the percent match is given in parentheses for the strand where cleavage was predicted but not observed. The column labeled "symmetry between sites" compares the relative efficiency of cleavage on each strand at companion sites; thus, "good" corresponds to equal intensity in bands derived from companion sites, whereas partial, poor, or none signifies that the intensities were unequal. [c] DNA fragments were uniquely end labeled at either the *Nco*I site or the *Ava*I site [−205 to −325 relative to the start site; see McGeoch et al. (1986)] and cleavage reactions performed with purified topoisomerase II (see Figures 2 and 3 for methods). Cleavage data are summarized from several different experiments using standardized conditions(DNA, topo II concentrations). On the sequence above, the cleavage sites are marked with vertical lines (the size of the line is proportional to intensity of cleavage indicated on the scale to the left of the sequence).

From differential end labeling, two strong cleavages on opposite strands were resolved (sites 1 and 2 in Figure 2 and Table III, experiment 1). Assuming that the single sites on each strand correspond to one cleavage event by the same enzyme molecule at the site, the results suggest a 5′ overhang of 4 bp is generated at the site, and this was verified by extension with T4 DNA polymerase (data not shown) (Sander & Hsieh, 1983). At sites 1 and 2, on opposing strands, the intensities of each cleavage product are roughly equal, suggesting that the probability of cleavage is approximately the same at each site. We refer to paired sites on opposing strands separated by 4 bp as "companion" sites. At some sites in the fragment, the cleavages were not of equal intensity on opposing strands. For example, sites A and B (bottom strand in Figure 2) were noticeably more intense than the G cleavages on the top strand (Table III). Moreover, we could not precisely align

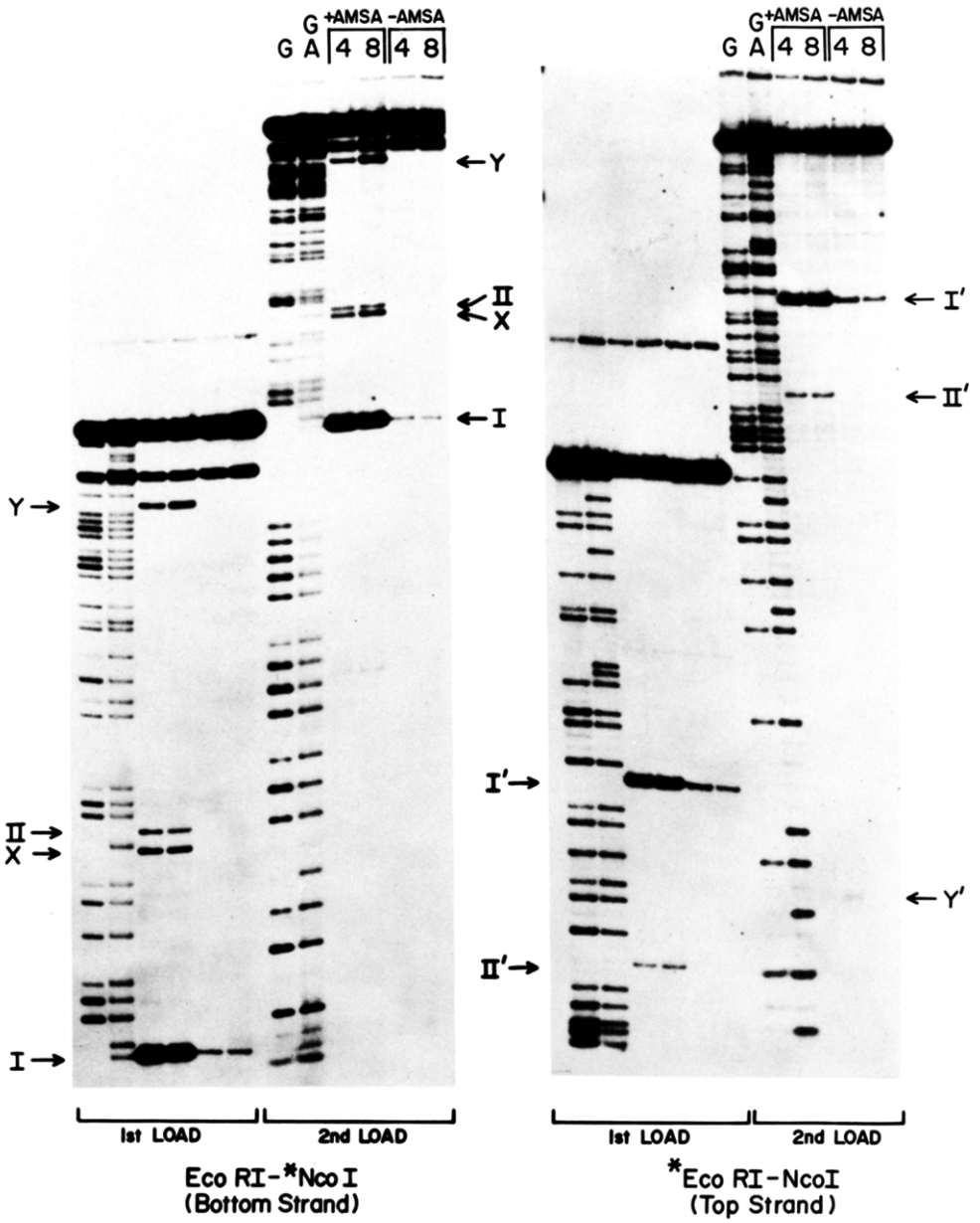


FIGURE 3: Topoisomerse II cleavages in the promoter of immediate early gene 3 of HSV-1. A 90-bp fragment was prepared from the promoter region of IE gene 3 of HSV-1 (*Eco*RI to *Nco*I, from −103 to −196, respectively, relative to the transcription start site) (Mackem & Roizman, 1980; McGeoch et al., 1986) and the DNA 5′ end labeled at either the *Eco*RI site (top strand) or the *Nco*I site (bottom strand). Cleavages were carried out as described in the legend to Figure 2 with either 4 or 8 units of topoisomerase II as indicated, and in the presence or absence of 10 μg of *m*-AMSA/mL. To resolve longer fragments, two staggered loadings were performed as indicated at the bottom of each gel. Sequence ladders were prepared by standard Maxam and Gilbert chemistry and are indicated at the top. The left panel shows the bottom-strand data and the right panel the top-strand data as marked. Symmetric topoisomerase II cleavage sites are designated by Roman numerals and asymmetric sites by letters the X and Y. The cleavages are shown on the DNA sequence in Table III (experiment 2). What appears to be an additional cleavage site immediately above Y is present in the sequencing ladders and is therefore independent of enzyme addition. Repeat experiments confirm that this site is not a strong topoisomerase II cleavage site (data not shown).

the B cleavage on the bottom strand with a companion site on the top strand. Similarly, at site C (lane 9, Figure 2) a companion site in the top strand was not detected. We refer to companion sites that are cleaved with equivalent frequency as "symmetric" sites (sites 1 and 2, Figure 2) whereas "asymmetric" sites are companion sites with unequal frequencies of cleavage (site C, Figure 2). These cleavage sites on the actual DNA sequence and a tabulation of the degree of symmetrical cleavage of companion sites are summarized in Table III.

To investigate the generality of this result, we tested cleavages on a DNA sequence that has a different G+C content than plasmid DNA. A promoter/enhancer region of the immediately early gene 3 (IE3) upstream region from HSV-1 (McGeoch et al., 1986; Mackem & Roizman, 1980; Lang et al., 1984) was 5′ end labeled (either top or bottom strand was tested) and reacted with topoisomerase II. Two symmetric cleavages were detected (marked I,I′ and II,II′) as well as two asymmetric sites (X and Y, Figure 3 and Table III). Sites X and Y correspond to relatively strong sites on the bottom strand; however, to detect the corresponding top-strand cleavages (4 bp upstream), extremely long exposures of the autoradiograph were required. A faint site is barely visible at Y′ in Figure 3 that aligns with the much stronger companion cleavage at Y; thus, the Y sites are defined as being asymmetric (see summary in Table III, experiment 2). The intense band immediately above site Y is not a strong topoisomerase II cleavage product, and repeat experiments have not detected a strong cleavage at this site (data not shown).

It is known that with end-labeled fragments cleavage

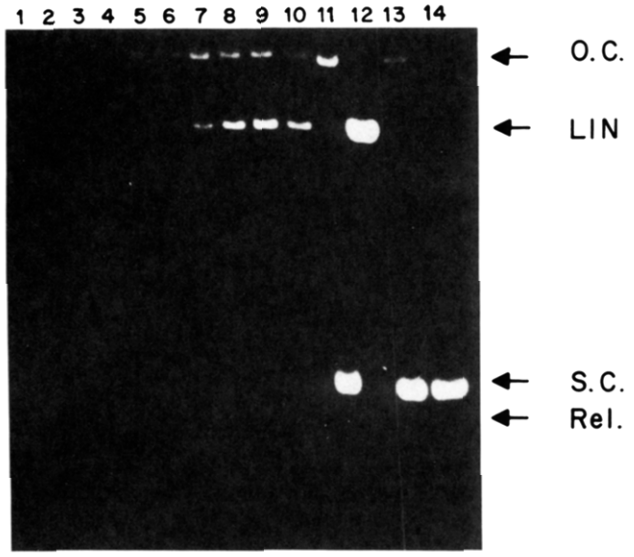


FIGURE 4: Analysis of DNA recovered from the SDS-K+ precipitate. Reactions were carried out in either topoisomerase II reaction buffer or topoisomerase I reaction buffer as specified for each enyzme. Reactions contained 200 ng of supercoiled plasmid DNA and were incubated for 30 min at 30 °C. All reactions were terminated with SDS. A 1% agarose gel containing 0.5 μg of ethidium bromide/mL was used to separate various DNA forms indicated to the right of the figure: O.C., nicked open circular DNA; Lin, linear DNA; S.C., supercoiled DNA; Rel. relaxed DNA. The reaction products shown in lanes 1–10 were recovered from the SDS-K+ precipitate [see preparative SDS-K+ method in Trask et al. (1984)] and correspond to those DNAs that are covalently bound to protein. Lane 1, negative control (no enzyme); lane 2, 2 units of topoisomerase I; lanes 3 and 4, 2 and 4 units of topoisomerase I, respectively, plus 0.25 mg of camptothecin/mL; lanes 5 and 6, 4 and 6 units of topoisomerase II, respectively, no drugs; lanes 7–10, 2, 4, 6, and 8 units of topoisomerase II, respectively, plus 10 μg of *m*-AMSA/mL; lane 11, open circular and supercoiled marker DNA positions; lane 12, a linear DNA marker; lanes 13 and 14, supercoiled DNA (DNA substrate used in reactions with topoisomerase II) with or without *m*-AMSA, respectively.

probabilities are profoundly influenced by strong sites which might truncate the fragment and remove the end label (thereby biasing downstream cleavages) (Lutter, 1978; Rhodes, 1985); however, it is unlikely that the observed cleavage differences at asymmetric sites can be explained in this way because we detected weak sites that were 5′ of the strong site. Furthermore, the A and B sites are further to the 3′ side of the strong site 2 (bottom strand in Figure 2); thus, one might predict that cleavages at A,B would be even more pronounced in the absence of strong site 2. This type of bias introduced from strong cleavage sites would be particularly serious if greater than one enyzme molecule is active per fragment; however, our current data (see Figure 4) tend to argue against this possibility at least under conditions used in our experiments. Collectively, the data suggest that two, single-stranded cleavages might proceed independently of each another; that is, either cleavage probabilities and/or resealing probabilities are independent events (see Discussion).

The suggestion that single cleavages are separable and not concurrent step predicts that single-stranded nicks should be detected when topoisomerase II reactions are interrupted with detergent. To test this, supercoiled plasmid DNA was reacted with topoisomerase II, the covalent DNA–enzyme intermediate was isolated by the preparative SDS-K+ precipitation method (Trask & Muller, 1983; Trask et al., 1984), and the DNA structure was determined by ethidium bromide agarose electrophoresis (Figure 4). Without *m*-AMSA, small amounts of linear and open circular DNA were recovered from the SDS-K+ precipitate (lanes 5 and 6). A control with topoisomerase I yielded low levels of open circular DNA in the SDS-K+ precipitate as expected (lanes 3 and 4). In topoisomerase II reactions containing *m*-AMSA, open circular and linear DNAs were increased 2–4-fold in the SDS-K+ precipitate (lanes 7–10). This stimulation is commensurate with the data in Table II, and the amount of linear DNA is roughly proportional to topoisomerase II in the reactions. The results are also consistent with the assertion that covalent complexes are being formed which have only single-stranded nicks. The latter suggests that topoisomerase II can be arrested in a complex containing one or more single-stranded nicks. Formation of open circular and linear complexes was stimulated by *m*-AMSA; however, the formation of linear complexes was proportional to enzyme concentration while the formation of open circular complexes was not.

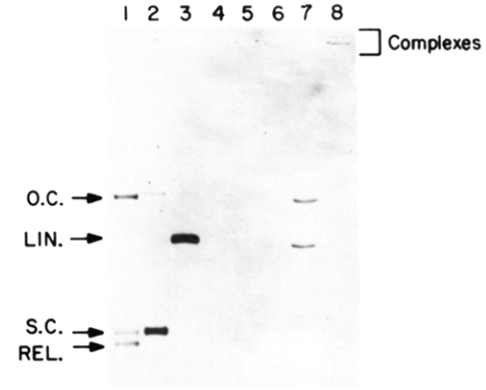


FIGURE 5: Nicked, open circular and linear DNAs contain stably bound protein. Reactions containing 300 ng of supercoiled plasmid DNA in topoisomerase II reaction buffer were incubated for 30 min at 30 °C and terminated with SDS. The reactions were then subjected to different treatments as follows. Lane 1 is a marker showing supercoiled, relaxed and open circular DNA. Lanes 2 and 3 are supercoiled and linear DNA markers, respectively. Reactions shown in lanes 4–8 were terminated with SDS and subjected to the SDS-K+ precipitation to recover covalent DNA–protein complexes: lane 4, a negative control (no topoisomerase II); lane 5, 0.5 unit of topoisomerase II (no *m*-AMSA); lane 6, 0.5 unit of topoisomerase II with 10 μg of *m*-AMSA/mL followed by digestion with proteinase K prior to SDS-K+ precipitation; lane 7, same as lane 6 except proteinase K digestion performed after SDS-K+ precipitation and recovery of complexes; lane 8, 0.5 units of topoisomerase II with 10 μg of *m*-AMSA/mL and SDS-K+ precipitation and recovery of complexes and no proteinase K digestion. The reaction products were analyzed on a 1.4% agarose gel containing 0.5 μg of ethidium bromide/mL. The various DNA forms are designated as in the legend to Figure 4. The position of covalent DNA–protein complexes is marked on the right side.

More rigorous proof that open circular DNA is stably associated with topoisomerase II is presented in Figure 5. SDS-K+-precipitable DNA was either undigested or digested with proteinase K prior to analysis on an ethidium bromide containing agarose gel. As shown in lane 7, roughly equal amounts of open circular and linear DNA were recovered from the SDS-K+ precipitate after proteinase K treatment. SDS-K+-precipitable DNA that was not treated with proteinase K was retained near the origin of the gel due to an association with protein (lane 8). Significantly, both linear and nicked open circular DNA forms were retained in the complex; therefore, it can be argued that open circular DNA is in fact stably (or covalently) bound to a protein and not adventitiously trapped as free DNA in the SDS-K+ precipitate by some unknown mechanism.

Analysis of Single-Strand Cleavages at the DNA Sequence Level. In order to determine whether single-strand cleavages occur at strong as well as weak topoisomerase II sites, the following experiment was performed. A fragment containing

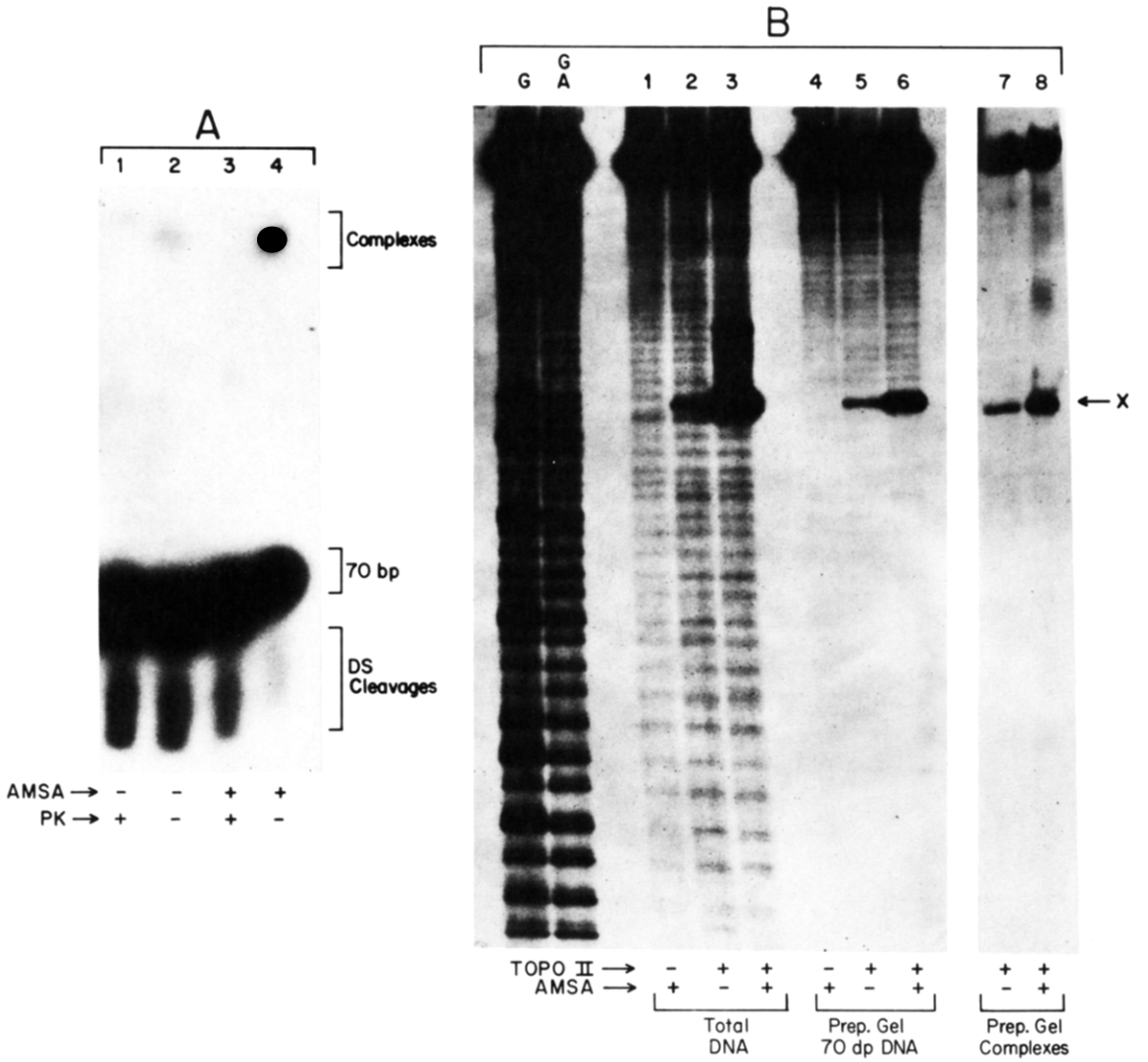


FIGURE 6: Double-stranded DNA fragments contain topoisomerase II induced single-strand nicks. A double-stranded oligonucleotide of 30 bp containing the strong topoisomerase II cleavage sites (sites 1 and 2, Figure 2, placed in the middle of the oligonucleotide) was cloned into the polylinker of pUC 12. A 70-bp fragment was prepared from this construction that was uniquely end labeled at a 5′ site to test cleavages at site 2 in Figure 2. Cleavage reactions were as specified in the legend to Figure 2. Reactions were terminated with SDS and treated with proteinase K. The indicated reactions were electrophoresed on a 5% native polyacrylamide gel, and the wet gel was exposed to film for 3 h (panel A): lanes 1 and 2 are reactions with 4 units of topoisomerase II without *m*-AMSA and after termination of reactions with SDS; lane 1 products were treated with proteinase K, and lane 2 products were untreated; lanes 3 and 4 each contained 4 units of topoisomerase II and 40 μg of *m*-AMSA/mL, and the reactions were either treated with proteinase K or untreated, respectively, before running the preparative gel. Restriction fragment marker positions are indicated to the left of panel A. The regions of the gel containing the double-stranded, intact 70-bp fragment and the complexes (marked with brackets) were each excised, were electroeluted, and after ethanol precipitation were loaded on a 20% sequencing gel shown in panel B. The chemical sequence ladders are shown in the left two lanes. Lane 1 is the negative control (70-bp fragment). Lanes 2 and 3 show topoisomerase II cleavage products (without and with *m*-AMSA, respectively) before loading the preparative gel. Lanes 4–8 were fragments recovered from the preparative gel. Lane 4 is a negative control that was not exposed to topoisomerase II. Lanes 5 and 6 show the cleavage sites in DNA recovered from the 70-bp fragment of reactions 1 and 3 of the gel shown in panel A. Lanes 7 and 8 show cleavages in the DNA recovered from the complexes which displayed reduced mobility in lanes 2 and 4 of the gel in panel A.

cleavage site 2 (see Table III, experiment 1) was end labeled and reacted with topoisomerase II in the presence and absence of *m*-AMSA. The resulting cleavage products were then separated on a preparative 5% native polyacrylamide gel to separate the intact 70-bp fragment from topoisomerase II DNA complexes and smaller fragments generated by double-strand cleavages (Figure 6A). As expected, the complexes displayed a retarded mobility (Figure 6A, lanes 2 and 4), were stimulated by *m*-AMSA, and were sensitive to proteinase K (compare lanes 1 and 2 or 3 and 4). The intact fragments and protein-associated DNAs were recovered from the preparative gel and analyzed on a 20% sequencing gel (Figure 6B). Lanes 2 (no drug) and 3 (with *m*-AMSA) are references for the strong topoisomerase II cleavages produced on the 70-bp fragment before running the preparative polyacrylamide gel. Lanes 4–8 correspond to fragments isolated from the preparative gel. In lane 4 the fragment was not incubated with topoisomerase II (negative control), showing that the fragment contains essentially no single-strand breaks. Lanes 5 (no drug) and 6 (with *m*-AMSA) show that the intact fragment recovered from the preparative gel contained single-stranded nicks (marked by an X). Furthermore, the single-stranded nicks occur at the same sites that were detected in the fragment prior to loading the preparative gel (compare lanes 2 and 3 with 5 and 6). Additional controls (lanes 7 and 8) show that the DNA associated with protein (labeled "complexes" in Figure 6A) contains the identical cleavage sequence.

Single-Strand Cleavages by Topoisomerase II Are Not Unique to Purified Enzyme. Topoisomerase II is a homodimer, and our data showing a single-strand cleavage product

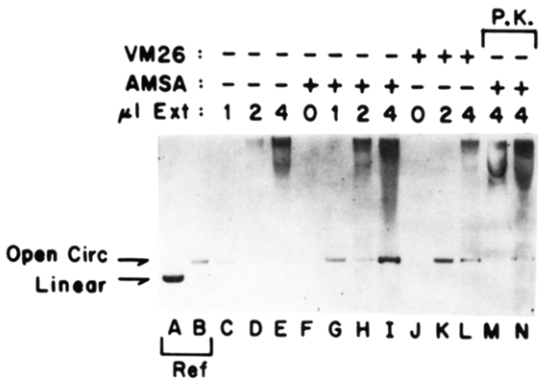


FIGURE 7: Topoisomerase II cleavages in a crude extract of yeast *top*1. The topoisomerase I deletion mutant, *top*1, was harvested as an exponentially growing culture. Spheroplasts, prepared according to standard methods, were resuspended (7 mL/g of cell pellet) in breakage buffer [0.7 M sorbitol, 0.05 M EDTA, 0.1 M Tris-HCl (pH 8.0), 0.14 M mercaptoethanol, 0.5 mM PMSF, 0.4 M KCl)]. An equal volume of glass beads (0.5-mm diameter) was added and the suspension shaken vigorously until spheroplasts were broken (5–6 times, 1 min each time). The lysate was first centrifuged for 5 min at 2000*g*. The supernatant was then centrifuged at 25000*g* for 30 min at 4 °C and assayed directly for topoisomerase II cleavages. Reactions were carried out in topoisomerase II reaction buffer (final volume of 40 μL) and contained 0.9 μg of supercoiled plasmid DNA plus the additions indicated below. The reactions were carried out at 30 °C for 30 min, terminated with SDS, and subjected to the preparative SDS-K+ procedure to recover those nucleic acids stably bound to protein. Lanes A and B, linear and open circular DNA markers, respectively; lanes C, D, and E, contained 1, 2, or 4 μL of the extract, respectively, and no addition of drugs; lanes F, G, H, I, and contained 0, 1, 2, or 4 μL, respectively, of extract and 25 μg of *m*-AMSA/mL; lanes J, K, and L, contained 0, 2, or 4 μL of extract, respectively, and 250 μg of VM-26/mL. In reactions C–L, the DNA was recovered from the SDS-K+ precipitate and digested with proteinase K prior to loading the 1% agarose–ethidium bromide gel. Lanes M and N are identical to lane I but were proteinase K treated in the following way. In lane M, the DNA from the SDS-K+ precipitate was not digested with proteinase K prior to loading the gel, and in lane N, the reaction products were digested with proteinase K *before* SDS-K+ precipitation. Note, the crude extract contained a rather large amount of nucleic acid, a small fraction of which was adventitiously trapped in the SDS-K+ precipitate. [This carry-over problem was proportional to the amount of extract (lanes F–I) and most notable with 4 μL of extract.]

could be explained by a mangled form of the enzyme (for example, one inactive subunit) that is not representative of the native activity. To address this issue required that we determine whether topoisomerase II has single-strand cleavage activity in an impure state, using freshly prepared extracts from cells. The experiment is complicated by high levels of topoisomerase I in crude extracts which also cause single-strand cleavages under conditions where topoisomerase II is active (data not shown). To avoid this complication, we performed the experiment using a topoisomerase I deletion mutant in yeast (*top*1) (Trash et al., 1984). Preliminary experiments with purified yeast topoisomerase II demonstrated that the yeast enzyme responds to *m*-AMSA and VM-26 like the avian enzyme (data not shown). A crude extract was freshly prepared from the *top*1 mutant and checked for the presence of topoisomerase II by ATP/$MgCl_2$-dependent relaxation of supercoiled DNA. To test for topoisomerase II mediated single-strand cleavages, we used the SDS-K+ assay to determine whether covalent DNA–protein complexes containing open circular DNA were formed under conditions predicted to be optimal for topoisomerase II cleavages (Figure 7). The SDS-K+ precipitate contained very low levels of open circular DNA in the absence of topoisomerase II inhibitors (lanes C, D, and E). In the presence of both *m*-AMSA and VM-26, the amount of open circular DNA associated with protein was increased by a factor of 3–10-fold (compare lanes C, D, and E with G, H, and I and with K and L). This level of stimulation is commensurate with previous data using purified topoisomerase II (see Table II). Controls demonstrate that the open circular DNA detected in these reactions was carried into the SDS-K+ precipitate because of stably (presumed covalently) bound protein: lane M shows that proteinase K treatment before SDS-K+ precipitation eliminated the open circular DNA; lane N shows that leaving out the proteinase K step after recovery of the SDS-K+-precipitated complexes (see Figure 7 legend) caused an electrophoretic shift in mobility of the open circular DNA band. Topoisomerase I is not present in these extracts; therefore, the SDS-K+-precipitated DNA–protein complexes cannot be due to the type I enzyme, and because the formation of SDS-resistant, open circular DNA–protein complexes was stimulated by *m*-AMSA and VM-26, we conclude that topoisomerase II is responsible.

DISCUSSION

The purification procedure described is relatively simple and yields a homogeneous enzyme preparation. Although chicken erythrocytes contain much less topoisomerase II per cell than MSB-1 tissue culture cells, erythrocytes are present in a 1000-fold higher concentration and are therefore a convenient source for starting material. We have also purified the enzyme from MSB-1 cells using this procedure, and the enzyme exhibits properties identical with enzyme from erythrocytes (Spitzner and Muller, unpublished observation).

Cleavage reactions were performed on a number of different end-labeled DNA fragments both in the presence and in the absence of topoisomerase II inhibitors which facilitate trapping of the enzyme in a covalent complex with DNA (Nelson et al., 1984; Tewey et al., 1984). The cleavage experiments revealed that detergent-arrested intermediates, containing covalently attached topoisomerase II, existed in at least two discernible forms. One was a double-stranded cleavage intermediate, and a second complex contained at least one single-stranded nick. The latter complexes were generated in a reaction between topoisomerase II and supercoiled plasmid DNA. The fact that open circular DNA was recovered directly from the SDS-K+ precipitate suggests that a protein was stably (or covalently) bound to the DNA (Trask et al., 1984); moreover, since the formation of open circular DNA was stimulated by *m*-AMSA, we conclude that topoisomerase II was attached to these DNA molecules and not a contaminating activity (like topoisomerase I).

Our data indicate that the double-stranded breakage and rejoining reaction, which unifies all type II enzymes, might be viewed as asymmetric single-stranded breakage and rejoining events, staggered by 4 bp at a site. The nicking (or resealing) steps on opposing strands do not appear to be concurrent and should be viewed as independent events with independent cleavage probabilities upon addition of detergent. Whether or not these independent cleavage probabilities are attributable to the detergent remains to be seen. The formation of nicked intermediates by eukaryotic topoisomerase II could also be explained by damaged enzyme molecules in the final fraction; however, we attach significance to the phenomenon because of reports showing that *m*-AMSA induces double- *and* single-stranded breaks in vivo (Ross et al., 1979; Zwelling et al., 1981) and in vitro (Minford et al., 1986). To determine whether the single-strand breaks are an aberrant reaction pathway caused by mangled enzyme, it was necessary to assay for topoisomerase II cleavages in crude extracts. Our experience is that topoisomerase II is a functionally conserved protein (Spitzner & Muller, 1988): Yeast topoisomerase II,

for example, recognizes the vertebrate topoisomerase II consensus sequence and is inhibited by the same drugs (data not shown). Thus, we used a fresh extract of a yeast mutant (deleted in topoisomerase I) (Thrash et al., 1984) and showed that topoisomerase II could be trapped in a nicked DNA enzyme complex. Surprisingly, we did not detect linear DNA cleavage products (Figure 7, lanes G, H, and I). The basis for this observation is unclear and may be caused by extraneous proteins in the crude extract. Alternatively, it is conceivable that the nicked intermediate is easier to trap with the native enzyme, whereas purified (perhaps modified) enzyme tends to produce double-strand cleavage intermediates. The experiment was repeated with chicken cell extracts, and we obtained similar results; viz., *m*-AMSA stimulated the formation of open circular DNA, although as noted above, high levels of topoisomerase I activity in the extracts were responsible for the formation of some open circular DNA. In addition, we have noticed that treatment of replicating SV40 DNA with VP16 in vivo stimulates the formation of predominantly open circular SV40 DNA as opposed to linear DNA (R. Snapka, V. Mehta, and M. Muller, unpublished results).

Additional pieces of data support our interpretation that single-stranded cleavages with topoisomerase II are independent and separable events. First, from sequencing around cleavages, we have identified single-stranded cleavage sites on each strand which cannot be aligned with a companion site (4 bp away) on the opposing strand. We interpret this to mean that at some sites single-strand cleavage intermediates were trapped, although most of the asymmetric sites aligned with weak topoisomerase II cleavage sites. Second, we considered the possibility that perhaps some cleavages occur on single-stranded pieces of DNA which might contaminate our fragments; however, we found that topoisomerase II does not cleave a single-stranded (synthetic) oligonucleotide containing the strong cleavage site (data not shown). We conclude that the single-stranded cleavages originated on double-stranded DNA.

In sum, the following observations support the idea that topoisomerase II is responsible for the single-stranded cleavages. First, the SDS-K+ precipitation method does not produce open circular DNA in the absence of topoisomerase II (Figure 4, lane 1, and Figure 5, lane 4) or under conditions where topoisomerase II activity is prevented. Second, formation of nicked DNA in the presence of the enzyme is increased by drugs (*m*-AMSA) that stimulate the cleavage reaction (Figure 4, lanes 5–10, and Figure 5, lanes 5 and 7). Third, proteinase K treatment of nicked DNA prior to SDS-K+ treatment prevents precipitation, showing that protein is associated with these molecules (Figure 5, lane 6). Fourth, essentially all nicked DNA molecules generated in the presence of topoisomerase II and isolated by SDS-K+ selection were found to be covalently bound to protein (Figure 5, lane 8). This was also true for linear DNA molecules. Fifth, identical results were obtained with several different, independently purified preparations of topoisomerase II. Sixth, we showed that topoisomerase II reactions arrested with SDS can result in a DNA product that contains single-stranded nicks and, furthermore, the sequence at the single-stranded cleavage site is the same as that identified in symmetric double-stranded DNA cleavages (compare site 2 in Figures 2 and 6). Finally, we showed that the single-strand nicks were stimulated by *m*-AMSA, which argues that topoisomerase II is responsible for this phenomenon (Figure 6, lanes 5 and 6).

A strong symmetric cleavage site (Figure 2, marked 1 and 2) along with 15 bp of 3′ and 5′ flanking DNA was synthesized as a 30-bp oligonucleotide which was then subcloned; therefore, this strong topoisomerase II site was imbedded into a different sequence background of vector DNA (outside the 30-bp oligonucleotide). The fact that site 2 was cleaved identically in this new sequence environment (compare Figures 2 and 6) demonstrates that a site plus 15 bp of 5′ and 15 bp of 3′ flanking DNA must contain appropriate recognition elements for topoisomerase II. These findings are consistent with our consensus sequence which defines the key residues over a span of 18 bp [(5′) −10 to +8 (3′) top strand] (Spitzner & Muller, 1988):

	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	1	2	3	4	5	6	7	8
5′	A/G	N	C/T	N	N	C	N	N	G	T/C	N	G	G/T	T	N	T/C	N	T/C
3′	T/C	N	G/A	N	N	G	N	N	C	A/G	N	C	C/A	A	N	A/G	N	A/G
	14	13	12	11	10	9	8	7	6	5	4	3	2	1	-1	-2	-3	-4

In this consensus sequence, N is any base, and cleavage occurs between +1 and −1. This consensus accurately predicts topoisomerase II sites in vitro when applied in the following manner: For each potential topoisomerase II site, both strands must be examined independently (taking into account the 4-bp 5′ overhang), and homology is scored by the number of matches to the "non-N" positions in the consensus sequence. The homology scores for both strands are summed, and a score of 11 or more consensus matches (out of 20 possible) predicts a topoisomerase II cleavage, while a score of 10 may be cleaved; however, the degree of homology (score) correlates roughly with strength of cleavage (see Figures 2 and 3 and Table III). Furthermore, sites with high scores clustered on one strand are cleaved more efficiently than sites with moderate scores distributed on both strands (compare band intensities for sites I and I′ with those for sites II and II′ in Figure 3; refer to Table III for homology to consensus sequence). Are consensus homologies important in defining single-strand or asymmetric cleavages? The data from three experiments are summarized in Table III. In general, we could not identify a clear-cut pattern relating consensus homology (top and bottom strands) and frequency of cleavage between companion sites. For example, in experiment 1, the symmetric sites 1 and 2 showed a 40% and 70% homology to the consensus, respectively, while other asymmetric sites also showed equivalent or even better top- and bottom-strand consensus homology (site B, site X, and site J in experiments 1, 2, and 3, respectively). These results show that simply comparing homology to the consensus sequence does not discriminate between symmetric and asymmetric cleavages by topoisomerase II; however, upon closer examination of the representative top- and bottom-strand cleaves (see homology summaries, Table III), clear differences were revealed. Specifically, all five of the sites displaying "good" symmetry (see explanation in Table III, footnote b) contain a T at the +4 position (or A at +1 on the complementary strand) on at least one of the DNA strands, and four of these five have a G residue at +2. In contrast, in the partially symmetric sites, only two of the six have a T at +4, and only one has both the +4 T and +2 G. Finally, in all the sites designated as "poor" or "none" in terms of symmetry, only two out of ten have a +4 T, and zero out of ten have both +4 T and +2 G. In an independent examination of another sequence (containing six cleavage sites in the *X. laevis* 5S rRNA gene), we found that the single good site also fit the +4 T and +2 G model; however, the remaining five sites were asymmetric to varying degrees (either "partial" or "none"), and all five sites lacked +4 T and +2G. These positions are important because the +4 T and +2 G are the two most conserved nucleotide positions in all topoisomerase II sites examined thus far (Spitzner & Muller, 1988). We conclude that all sequences with threshold homology to the consensus sequence above will

be cleaved by topoisomerase II; however, whether a given sequence will yield single- vs double-strand cleavage products depends upon the composition at the +4 and +2 positions.

Our observation that topoisomerase II can produce single-strand cleavages might explain how topoisomerase II could complement a mutation in topoisomerase I (Uemura & Yanagida, 1984; Brill et al., 1987). If complementation of a defect in topoisomerase I involves the action of topoisomerase II at (or near) a topo I recognition site, the question arises as to what elements are directing or attracting topoisomerase II. Since we have been unable to demonstrate common sequence recognition elements for topoisomerases I and II (Spitzner, Trask and Muller, unpublished observation), it appears that sequence recognition alone cannot explain why topoisomerase II might act at topo I sites in chromatin, if indeed it does. Furthermore, it is not known whether the breaks in DNA made by topoisomerase II (in wild-type or mutant cells) in vivo are actually single stranded, double stranded, or both in the absence of drugs or detergents because the phenomenon has not been observed in vivo in the absence of either of these.

ACKNOWLEDGMENTS

Anti-topoisomerase II antibody was a gift from M. Heck and W. Earnshaw for which we are grateful. The yeast mutant, *top*1, was kindly provided by R. Sternglanz.

Registry No. DNA topoisomerase, 80449-01-0.

REFERENCES

Baldi, M. I., Benedetti, P., Mattoccia, E., & Tocchini-Valentini, G. P. (1980) *Cell (Cambridge, Mass.) 20*, 461–467.

Benedetti, P., Baldi, M. I., Mattoccia, E., & Tocchini-Valentini, G. P. (1983) *EMBO J. 2*, 1303–1308.

Berrios, M., Osheroff, N., & Fisher, P. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4142–4146.

Brill, S. J., DiNardo, S., Voelkel-Meiman, K., & Sternglanz, R. (1987) *Nature (London) 316*, 414–416.

Champoux, J. J., & Dulbecco, R. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 143–146.

DiNardo, S., Voelkel, K., & Sternglanz, R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2616–2620.

Duguet, M., Lavenot, C., Harper, F., Mirambeau, G., & De Recondo, A. (1983) *Nucleic Acids Res. 11*, 1059–1075.

Duoc-Rasy, S., Kayser, A., Riou, J.-F., & Riou, G. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7152–7156.

Dynan, W. S., Jendrisak, J. J., Hager, D. A., & Burgess, R. R. (1981) *J. Biol. Chem. 256*, 5860–5865.

Earnshaw, W. C., & Heck, M. S. (1985) *J. Cell Biol. 100*, 1716–1725.

Gasser, S. M., Laroche, T., Falquet, J., de la Tour, E. B., & Laemmli, U. K. (1986) *J. Mol. Biol. 188*, 613–629.

Goto, T., & Wang, J. C. (1982) *J. Biol. Chem. 257*, 5865–5872.

Hajduk, S. L., Klein, V. A., & Englund, P. T. (1984) *Cell (Cambridge, Mass.) 36*, 483–492.

Holm, C., Goto, T., Wang, J. C., & Botstein, D. (1985) *Cell (Cambridge, Mass.) 41*, 553–563.

Hsieh, T., & Brutlag, D. (1980) *Cell (Cambridge, Mass.) 21*, 115–125.

Jendrisak, J. J., & Burgess, R. R. (1975) *Biochemistry 14*, 4639–4645.

Lang, J. C., Spandidos, C., & Wilkie, N. M. (1984) *EMBO J. 13*, 389–395.

Liu, L. F., Rowe, T. C., Yang, L., Tewey, K. M., & Chen, G. L. (1983) *J. Biol. Chem. 258*, 15365–15370.

Lutter, L. C. (1978) *J. Mol. Biol. 124*, 391–420.

Mackem, S., & Roizman, B. (1980) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4917–4921.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Marini, J. C., Miller, K. G., & Englund, P. T. (1980) *J. Biol. Chem. 225*, 4976–4979.

Maxam, A. M., & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 564.

McGeoch, D. J., Dolan, A., Donald, S., & Brauer, D. H. K. (1986) *Nucleic Acids Res. 14*, 1727–1745.

Miller, K. G., Liu, L. F., & Englund, P. T. (1981) *J. Biol. Chem. 256*, 9334–9339.

Minford, J., Pommier, Y., Filipski, J., Kohn, K. W., Kerrigan, D., Mattern, M., Michaels, S., Schwartz, R., & Zwelling, L. A. (1986) *Biochemistry 25*, 9–16.

Nelson, E. M., Tewey, K. M., & Liu, L. F. (1984) *Proc. Natl. Acad.* Sci. *U.S.A. 81*, 1361–1365.

Rhodes, D. (1985) *EMBO J. 4*, 3473–3482.

Ross, W. E., Glaubiger, D. E., & Kohn, K. W. (1979) *Biochim. Biophys. Acta 562*, 41–50.

Sander, M., & Hsieh, T. (1983) *J. Biol. Chem. 258*, 8421–8428.

Saucier, J. M., Benard, J., DaSilva, J., & Riou, G. (1981) *Biochem. Biophys. Res. Commun. 101*, 988–994.

Shelton, E. R., Osheroff, N., & Brutlag, D. L. (1983) *J. Biol. Chem. 258*, 9530–9535.

Spitzner, J. R., & Muller, M. T. (1988) *Nucleic Acids Res. 16*, 5533–5556.

Tewey, K. M., Chen, G. C., Nelson, E. M., & Liu, L. F. (1984) *J. Biol. Chem. 259*, 9182–9187.

Thrash, C., Voelkel, K., DiNardo, S., & Sternglanz, R. (1984) *J. Biol. Chem. 259*, 1375–1377.

Trask, D. K., & Muller, M. T. (1983) *Nucleic Acids Res. 11*, 2779–2800.

Trask, D. K., DiDonato, J. D., & Muller, M. T. (1984) *EMBO J. 3*, 671–676.

Tsutsui, K., Tsutsui, K., Sakurai, H., Shohmori, T., & Oda, T. (1986) *Biochem. Biophys. Res. Commun. 138*, 1116–1122.

Udvardy, A., Shedl, P., Sander, M., & Hsieh, T. (1986) *J. Mol. Biol. 191*, 231–246.

Uemura, T., & Yanagida, M. (1984) *EMBO J. 3*, 1737–1744.

Uemura, T., & Yanagida, M. (1986) *EMBO J. 5*, 1003–1010.

Vosberg, H. P. (1985) *Curr. Top. Microbiol. Immunol. 114*, 19–101.

Wang, J. C. (1985) *Annu. Rev. Biochem. 54*, 669–697.

Yanisch-Perron, C., Vieira, J., & Messing, J. (1985) *Gene 33*, 103–119.

Zwelling, L. A., Michaels, S., Erickson, L. C., Ungerleider, R. S., Nichols, M., & Kohn, K. W. (1981) *Biochemistry 20*, 6553–6563.