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# DNase I Hypersensitivity Is Independent of Endogenous Topoisomerase II Activity during Chicken Erythrocyte Differentiation

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**Endogenous topoisomerase II cleavage sites were mapped in the chicken $\beta^A$-globin gene of 12- to 14-day embryonic erythrocytes. A major topoisomerase II catalytic site was mapped to the 5′ end of the globin gene which contained a nucleosome-free and DNase I-hypersensitive site and additional but minor sites were mapped to the second intron and 3′ of the gene to a tissue-specific enhancer. Cleavage sites, mapped in situ by indirect end labeling, were aligned to single-base-pair resolution by comparison to a consensus sequence derived for vertebrate topoisomerase II catalytic sites. In contrast to embryonic erythrocytes, endogenous topoisomerase II cleavages were not detected in erythrocytes from peripheral blood of adult chickens; therefore, as the transcriptional activity of the $\beta^A$-globin gene declines during terminal differentiation of erythrocytes, the activity of topoisomerase II in situ declines as well, despite the fact that DNase I hypersensitivity persists. The results showed that DNase I-hypersensitive chromatin can be maintained in the absence of topoisomerase II activity and suggested that topoisomerase II acts at hypersensitive sites because of an inherent attraction to some preexisting combination of DNA sequence or chromatin structure associated with DNase I-hypersensitive regions.**

The physiological function of eucaryotic topoisomerase II has been partially established. The enzyme is required for chromosome segregation in *Saccharomyces cerevisiae* (8, 19, 50) and presumably in higher eucaryotes as well, since its activity shows cell cycle fluctuation in both yeast and animal cells (8, 10, 16a, 47, 51). Additionally, topoisomerase II has been implicated in transcription on the basis of an association with transcriptionally active chromatin (38, 48, 53, 54, 62) and its ability to complement topoisomerase I in a yeast topoisomerase I deletion mutant (3). A direct demonstration that the two types of topoisomerase are interchangeable, at least in the relaxation of plasmid DNA in vivo, comes from studies showing that either enzyme can substitute for the other in yeast cells (40).

There has been some speculation that topoisomerase II may be important in the organization of active chromatin that is torsionally stressed, as suggested by studies on 5S chromatin in vivo and in vitro (16, 23, 39); however, the existence of eucaryotic gyrase and torsional strain in eucaryotic DNA is controversial (57, 59), and it is not clear whether eucaryotic cells interconvert torsional twist and axial writhe like bacteria (12, 25, 62). The similarities between topoisomerase II and bacterial DNA gyrase have been noted (26, 41), but purified topoisomerase II clearly lacks the ability to decrease linking number (i.e., introduce negative supercoils) in vitro. The positioning of topoisomerase II at the base of loop domains in the nuclear matrix-chromosomal scaffold (2, 11) places it at a strategic site for influencing domain topology (15) and might also implicate topoisomerase II in DNA replication, as suggested in previous reports (3, 32). Indeed, the nuclear matrix-scaffold has been reported to serve as a fixed site for DNA replication, which means that topoisomerase II is physically located near replication machinery such as DNA polymerase alpha and primase (21, 58). We recently derived a consensus sequence for vertebrate type II topoisomerases and noted that enhancer sequences are remarkably enriched in the consensus sequence (42a). This finding alone suggested that topoisomerase II exerts some influence over transcriptional activity; however, transcriptional regulatory sequences (promoters, enhancers) can influence DNA replication (1, 4, 7, 17, 24, 52), which again implicates topoisomerase II in DNA replication. Topoisomerase II could be important in both transcription and replication if topoisomerase II is involved in establishing or maintaining DNase hypersensitivity which is common to both processes. DNase-hypersensitive regions apparently mark origins of DNA replication (35; for reviews, see references 12 and 36).

Endogenous topoisomerase II sites have been mapped in situ at DNase I-hypersensitive sites (38, 61). If topoisomerase II acts at these sites before formation of DNase I hypersensitivity, a case could be made for proposing that topoisomerase II and nuclease hypersensitivity are functionally related. In this report, we explore the relationships among identifiable topoisomerase II sites in chromatin and DNase I-hypersensitive sites in vivo during differentiation of the chicken erythrocyte. In this system, we can compare the $\beta^A$-globin gene in a DNase I-hypersensitive chromatin structure in cells that are transcriptionally active (embryos) versus cells that are inactive (adults).

## MATERIALS AND METHODS

**Cultivation of cells and isolation of erythrocytes.** Erythrocytes from 12- to 14-day chicken embryos were obtained by nicking an amniotic blood vessel and recovering the blood with a 21-gauge needle and 1-cc syringe, followed by washing with buffer A (10 mM Tris hydrochloride [pH 7.6], 0.15 M NaCl) until all amniotic fluid was removed. Mature erythrocytes were obtained by wing bleeding adult birds. MSB-1 cells, a lymphocyte line transformed by Marek's disease virus, were propagated in suspension culture by using RPMI 1640 medium supplemented with 10% calf

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serum, and the cells were grown under 5% $CO_2$ tension at 41°C.

**Detection and isolation of topoisomerase-DNA complexes.** After the cells were washed in buffer A, the final pellet was suspended in 4 to 6 pellet volumes of buffer B (50 mM Tris hydrochloride, 1 mM EDTA, 5 mM $MgCl_2$, 0.5% Nonidet P-40) and incubated on ice for 10 min. Nuclei were released with five to six strokes with the A pestle of a Dounce homogenizer, and the mixture was centrifuged for 5 min at 1,500 × *g*, washed again with five-pellet volumes of buffer B, and suspended to 5 × $10^8$/ml in buffer B2 (20 mM Tris hydrochloride [pH 8.0], 1.5 mM $CaCl_2$, 3 mM ATP, 60 mM KCl, 3 mM β-mercaptoethanol). Topoisomerase II-DNA covalent complexes were trapped by incubating isolated nuclei in buffer B2 at 37°C for 30 min, followed by the addition of sarcosyl to 1% (vol/vol) and rapid mixing. Then, 2 volumes of 9 M NaSCN were added, and the lysate was layered on a step CsCl gradient (42) which was centrifuged in an SW41 rotor (33,000 rpm, 18 h) and fractionated into 0.4-ml fractions. The DNA peak was localized by determining the absorbance at 260 nm, pooled, dialyzed against buffer C (10 mM Tris hydrochloride [pH 7.6], 1 mM EDTA), and digested with restriction enzymes as specified. If the topoisomerase-DNA complexes were to be analyzed by the sodium dodecyl sulfate (SDS)-$K^+$ precipitation method (45, 46), SDS was added to 1%, followed by the addition of 0.1 volume of 2.5 M KCl. After 15 min on ice, the precipitate was deposited by centrifugation (12,000 × *g*, 2 min). The pellet was washed three times by suspension in 1 ml of buffer D (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA, 100 mM KCl), and the centrifugation step was repeated. The final pellet was suspended in buffer E (same as buffer D, with NaCl replacing KCl). The DNA was precipitated with ethanol, washed with 70% ethanol, and suspended in buffer E followed by digestion with proteinase K (50 μg/ml) for 30 min at 60°C.

**Immunoblot assays.** Antigens were either purified topoisomerases or fractions from step CsCl gradients, as indicated for each experiment. Gradient fractions were diluted to a final volume of 0.4 ml in buffer E (to reduce the salt concentration to less than 0.5 M) and then were applied to a nitrocellulose filter which had been presoaked in 150 mM NaCl–15 mM sodium citrate (pH 7.0). Each sample well was washed with 0.4 ml of 25 mM sodium phosphate (pH 6.5), after which the filter was air dried. The nonspecific binding sites were blocked by treatment with 3% gelatin in buffer F (20 mM Tris hydrochloride [pH 7.5], 0.5 M NaCl). The filter was incubated with anti-topoisomerase II immunoglobulin G (a monoclonal antibody provided by W. Earnshaw) diluted 1:600 in buffer F containing 1% gelatin. The filter was then washed several times with gentle agitation in buffer F, containing 1% gelatin, and labeled with 2.5 μCi of iodinated protein A (44).

**Analysis of DNA cleavages.** Reaction experiments were carried out in a final volume of 20 μl in a cleavage buffer containing the following final concentrations of solutes: 20 mM Tris hydrochloride (pH 8.0), 3 mM mercaptoethanol, 4 mM $MgCl_2$, 100 mM NaCl, 3 mM ATP, 0.1 mg of bovine serum albumin per ml. Where indicated, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) was added to the reaction just before the addition of topoisomerase II. The final concentration of *m*-AMSA is indicated in each experiment. Reaction mixtures were incubated at 30°C for 20 min and were stopped by the addition of 2 volumes of 1.5% SDS followed by digestion with proteinase K as described above. After the addition of 0.3 volume of 4.5 M ammonium acetate, the DNA was ethanol precipitated and washed with 70% ethanol, and the pellet was suspended in buffer E. The DNA was mixed with 0.1 volume of a dye-glycerol stock (0.05% bromophenol blue and 50% glycerol) before being loaded on a 1% agarose gel. Electrophoresis was performed at 2 V/cm in TAE buffer (40 mM Tris, 25 mM sodium acetate, 1 mM EDTA [pH 8.3]) containing 0.5 μg of ethidium bromide per ml; then the gel was dried and exposed to film.

**Miscellaneous procedures.** Preparation of plasmid DNA and Southern blot hybridizations were performed by using procedures described by Maniatis et al. (27). Radioactive DNA probes were prepared by nick translation as previously described (27). End-labeled DNA fragments were prepared by using polynucleotide kinase and [$^{32}$P]ATP to label the 5′ ends (27, 28).

## RESULTS

**Optimal conditions for cross-linking topoisomerase II and DNA in situ.** Topoisomerase II was trapped in a covalent complex with DNA in situ by incubating nuclei under conditions which favor catalytic activity of the endogenous enzyme, followed by lysis with a protein denaturant to arrest the covalent intermediate and produce a double-strand cleavage; a similar strategy was used previously to cross-link topoisomerase I (30). Topoisomerase II was more difficult to trap in a covalent complex with DNA compared with topoisomerase I, and it was necessary to use topoisomerase II inhibitors (5, 31) to facilitate the formation of a cleavable complex. Optimal conditions for cross-linking topoisomerase II in situ also required a buffer that was different from the typical reaction buffer used to assay the enzyme in vitro. Cross-linking was measured by the amount of topoisomerase II in the DNA peak of the gradient as quantified by immunoblotting. Figure 1 (two panels) shows that $CaCl_2$ and ATP in the presence of *m*-AMSA or teniposide (VM-26) were required for the formation of covalent topoisomerase II-DNA complexes. $MgCl_2$ was a poor substitute for $CaCl_2$ either in the presence or absence of drugs and ATP. In all cases, ATP stimulated the formation of covalent complexes. In vitro, calcium has been reported to trap the covalent topoisomerase II-DNA intermediate (34), and these findings were reflected in vivo as well. Figure 1 also shows a typical CsCl gradient profile of the distribution of free and bound topoisomerase II. Under optimal conditions (VM-26 or *m*-AMSA in buffer B2), about 5 to 10% of the total topoisomerase II was trapped in a covalent complex, compared with 25 to 30% with topoisomerase I (latter data not shown).

**Mapping topoisomerase II sites in active genes.** When 14-day erythrocyte nuclei were incubated under conditions optimal for topoisomerase II cross-linking in vivo, an *m*-AMSA-dependent cleavage was detected in the $\beta^A$-globin gene region (Fig. 2). The cleavage site was mapped by hybridization with a probe derived from the 3′ end of the *Eco*RI fragment (*Sma*I to *Eco*RI, see probe B in Fig. 4). The results of the indirect end-labeling experiments showed that (i) cleavage was dependent on the addition of topoisomerase II inhibitors and in all cases the appearance of the 5-kilobase (kb) band correlated with the detection of topoisomerase II in the DNA peak of the CsCl gradients; (ii) the intensity of the 5-kb band was significantly reduced when DNA from the CsCl step gradient was not exposed to proteinase K (Fig. 2, lane 5), compared with the same DNA digested with proteinase K before running the agarose gel (lane 6), suggesting that a protein is linked to the DNA in the 5-kb fragment which caused a shift in electrophoretic mobility; (iii) the

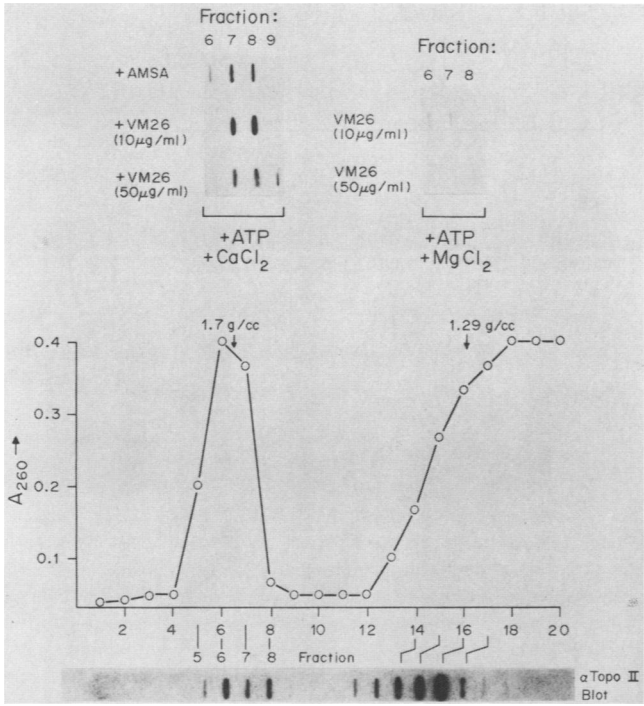


FIG. 1. Conditions for trapping the covalent topoisomerase II-DNA intermediate. Nuclei were prepared from exponentially growing MSB-1 cells as described in Materials and Methods and were suspended to $5 \times 10^8$/ml in the standard topoisomerase II buffer (buffer B2, defined in Materials and Methods) or a modified buffer B2, with 1.5 mM $MgCl_2$ replacing $CaCl_2$ (all other solutes identical). The reaction mixtures (containing $2 \times 10^8$ nuclei each) were incubated at 37°C for 15 min and treated with sarcosyl (1% final); the lysates were diluted with NaSCN and centrifuged in step CsCl gradients. The inset photographs at the top show the amount of topoisomerase II detected in the DNA peak fractions of the gradients as measured by immunoblotting with anti-topoisomerase II immunoglobulin G. The left inset shows reaction experiments in B2 buffer in addition to the following: top row, plus *m*-AMSA (50 μg/ml); middle row, plus VM-26 (10 μg/ml); bottom row, plus VM-26 (50 μg/ml). The right inset shows reaction experiments in modified B2 containing $MgCl_2$ in place of $CaCl_2$. The graph shows the results of a separate experiment in which nuclei were incubated under standard conditions (B2 buffer plus 50 μg of *m*-AMSA per ml) and were analyzed on the CsCl gradient, followed by Western immunoblotting with anti-topoisomerase II immunoglobulin G to localize the antigen in the gradient.

cleavage maps within 200 base pairs 5′ of the $\beta^A$-globin gene, and additional (but faint) bands were seen (bracket by lane 6, characterized below).

**Topoisomerase II cleavages in different tissue settings.** In chicken erythrocytes, the $\beta^A$-globin gene is transcribed in 12- to 14-day embryos but not in other tissues or in erythrocytes taken from the peripheral blood of adult birds (37, 43). (We refer to the latter as adult erythrocytes.) Endogenous topoisomerase II cleavage sites were not detected in adult erythrocytes or embryonic brain cells (Fig. 3). Nuclei from embryonic erythrocytes clearly showed the *m*-AMSA-dependent bands that flank the 5′ and 3′ regions of the coding sequence of the $\beta^A$-globin gene (sites labeled A1, A2, and A3). The most prominent cleavage (site A1) mapped to the nuclease hypersensitivity region identified by McGhee et al. (29). The autoradiogram shown in Fig. 3 was overexposed to reveal minor bands that might exist in either adult or brain nuclei. Additional tissues and different genes were also

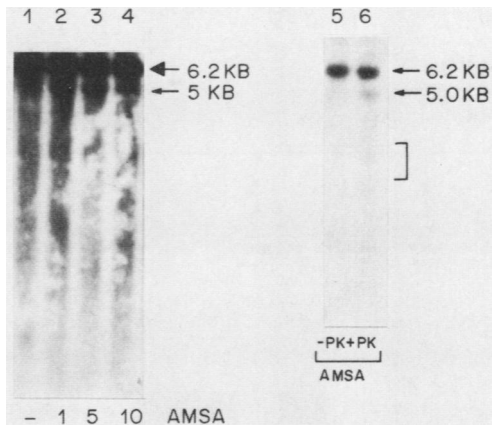


FIG. 2. *m*-AMSA induced double-stranded cleavages in nuclei. Nuclei were prepared from erythrocytes taken from embryos approximately 12 to 14 days into development. The nuclei were incubated and lysed with detergent to induce covalent complexes, followed by CsCl gradient centrifugation and collection of the DNA-containing fractions. The DNA was dialyzed and digested with *Eco*RI and analyzed by Southern blotting, using probe B shown in Fig. 4. Lanes: 1 to 4, nuclei were incubated in B2 buffer containing either 0, 1, 5, or 10 μg of *m*-AMSA (respectively) per reaction; 5 and 6, reaction experiments were carried out exactly as described for that in lane 4 except that after restricting with *Eco*RI, the DNA was divided into two fractions, one was treated with proteinase K (+PK) (50 μg/ml for 30 min at 60°C) and the other was not treated with proteinase K (−PK).

evaluated. In MSB-1 cells for example, we did not detect the *m*-AMSA-dependent cleavage sites in the adult $\beta^A$-globin gene region; however, we did find them in another active gene, cytochrome C (V. Mehta and M. Muller, unpublished data). The prominent site in cytochrome C mapped considerably further 5′ of the gene than did the $\beta^A$-globin site in embryonic erythrocytes and was also near a prominent DNase I-hypersensitive site (data not shown).

To further characterize the topoisomerase II cleavage site, several control experiments were performed to show that the cleavages were actually mediated by topoisomerase II. As shown in Fig. 4A, the formation of the 5-kb fragment (site A1 in Fig. 3) was reduced by the addition of 0.5 M NaCl before adding detergent. Similarly, adding EDTA before sarcosyl lysis resulted in a decrease in the yield of cleavage products (data not shown). Both treatments can reverse formation of a cleavable topoisomerase II-DNA complex (26, 41, 45, 48). To rule out the possibility that topoisomerase I is responsible for a double-stranded cleavage event (for example, topoisomerase I sites on opposing strands generating a double-stranded cleavage), the topoisomerase II inhibitor was replaced with the topoisomerase I inhibitor, camptothecin (20), in a reaction experiment carried out under conditions which trap topoisomerase I complexes (30). In contrast to *m*-AMSA and VM-26, the topoisomerase I inhibitor did not stimulate cleavage or formation of any secondary bands. Parenthetically, topoisomerase I is active in the $\beta^A$-globin gene in 12-day erythrocytes (M. Muller, and D. Trask, unpublished observations); however, the topoisomerase I intermediates contain single-stranded nicks (30). Collectively, the data strongly suggest that topoisomerase II was responsible for double-stranded cleavages under conditions defined in these assays.

Additional confirmation that there was a difference between topoisomerase II catalytic activity in embryonic ver-

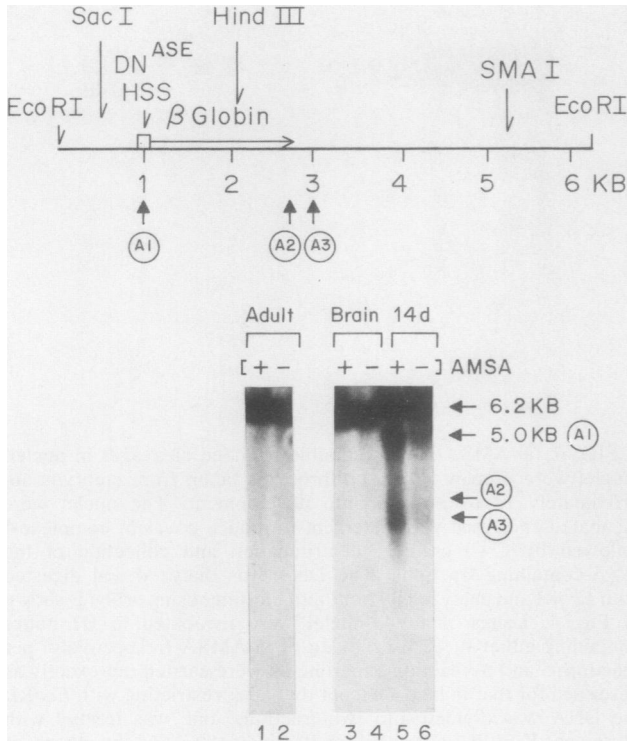


FIG. 3. Mapping topoisomerase II sites in different tissues. The in situ assay for topoisomerase II cleavage sites was performed as described in the legend to Fig. 2. Southern blots were hybridized to probe B (Fig. 4). Nuclei were prepared from adult erythrocytes (lanes 1 and 2), 12- to 14-day embryonic brain cells (lanes 3 and 4), or 12- to 14-day embryonic erythrocytes (lanes 5 and 6) and were incubated in the presence or absence of *m*-AMSA as indicated. The restriction map shows the positions of cleavage sites (marked A1 to A3), and the DNase-hypersensitive sites (HSS) are marked.

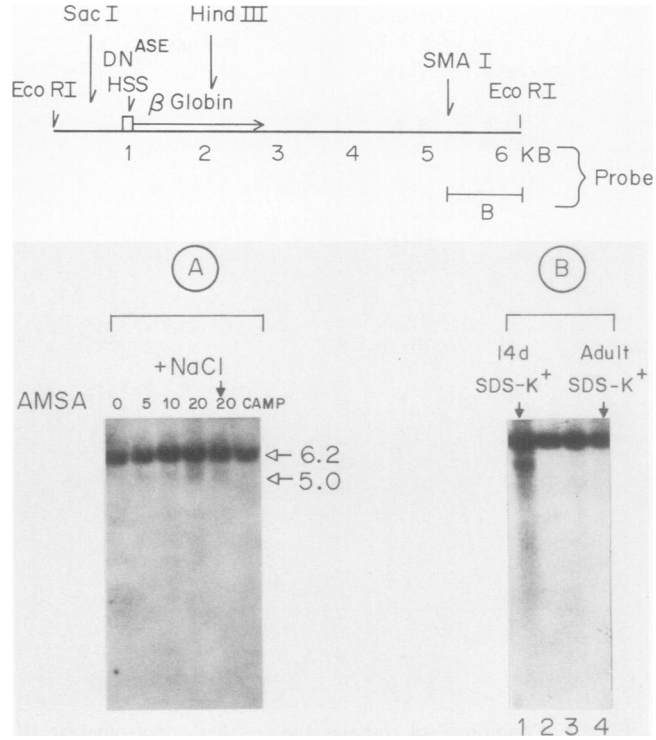


FIG. 4. Recovery of topoisomerase-DNA complexes by SDS-K+ precipitation. Nuclei were prepared from either 14-day embryonic or adult erythrocytes and were incubated under conditions described below before being cross-linked with detergent and CsCl gradient purification of the DNA. The DNA was digested with *Eco*RI and analyzed by Southern blotting, using probe B shown in the map (*Sma*I to *Eco*RI). (A) Nuclei ($2 \times 10^8$) from embryonic erythrocytes were incubated (15 min at 37°C) in the presence of (from left to right) 0, 5, 10, or 20 μg of *m*-AMSA per reaction. The lane labeled "+NaCl" was a reaction experiment incubated with 20 μg of *m*-AMSA per ml, and NaCl was added to a final concentration of 0.5 M before the addition of detergent. Lane CAMP was a reaction experiment incubated with 250 μg of camptothecin (lactone form per ml) for 15 min at 37°C. (B) Identical numbers of nuclei ($2 \times 10^8$) from either 14-day embryonic erythrocytes (lanes 1 and 2) or adult erythrocytes (lanes 3 and 4) were incubated in buffer B2 containing 10 μg of *m*-AMSA and then were lysed with detergent. The DNA was purified from the CsCl gradient, restricted with *Eco*RI, and subjected to SDS-K+ precipitation as described in Materials and Methods. The SDS-K+ precipitates and supernatants were ethanol precipitated and then digested with proteinase K (50 μg/ml for 1 h at 60°C). In each sample, the entire SDS-K+ precipitate was loaded on a 1% agarose gel. The supernatant DNA (10% of the total) was loaded in adjacent lanes of the same gel. Lanes 1 and 2, 14-day embryonic nuclei, SDS-K+ precipitable DNA, and supernatant DNA, respectively; lanes 3 and 4, adult erythrocyte DNA, supernatant, and SDS-K+ precipitable DNA, respectively.

sus adult erythrocytes was achieved in the following experiment. Covalent DNA-protein complexes were isolated from embryonic or adult nuclei by using the SDS-K+ method (45, 46) and then were characterized by Southern blotting to determine whether the DNA fragments contained features of a topoisomerase II cleavage intermediate. The experiment was quantitative because the concentrations of nuclei were identical in the two reaction mixtures and the relative concentration of covalent DNA-topoisomerase II complexes was measured in the same experiment. The DNA was purified, and after digestion with *Eco*RI, DNA fragments were fractioned into SDS-K+ precipitable DNA (to recover protein-bound DNA complexes) and supernatant (protein-free) DNA. A Southern blot analysis of these DNAs (Fig. 4B) revealed that embryonic DNA (lane 1) contained covalent complexes with characteristics of topoisomerase II (sites A1, A2, and A3 in Fig. 2), whereas DNA from adult erythrocytes (lane 4) was missing these bands. Longer exposures of the blot did not reveal additional bands from adult tissue. As noted above, conditions for trapping topoisomerase II in embryonic erythrocytes (Fig. 1) also trap topoisomerase I which is acting in this gene region (Muller and Trask, unpublished); thus, the SDS-K+ precipitate additionally contained DNA fragments that were covalently coupled to topoisomerase I (lane 1), and these DNA fragments are all parental size because type I topoisomerase makes single-stranded nicks in the DNA intermediate.

**DNase I hypersensitivity in the $\beta^A$-globin gene.** DNase I hypersensitivity is usually correlated with transcription (55); however, hypersensitive sites can be detected in genes that are transcriptionally quiescent (43, 56, 60), such as the $\beta^A$-globin gene in adult erythrocytes of mature birds. Although this gene is transcriptionally inactive, it nonetheless has DNase I-hypersensitive sites presumably reflecting transcription of the gene at an earlier stage in development. DNase I-hypersensitive sites were mapped in 5′- and 3′-end flanking regions of the $\beta^A$-globin gene in embryonic (12- to 14-day) and adult erythrocytes (Fig. 5), and the two patterns

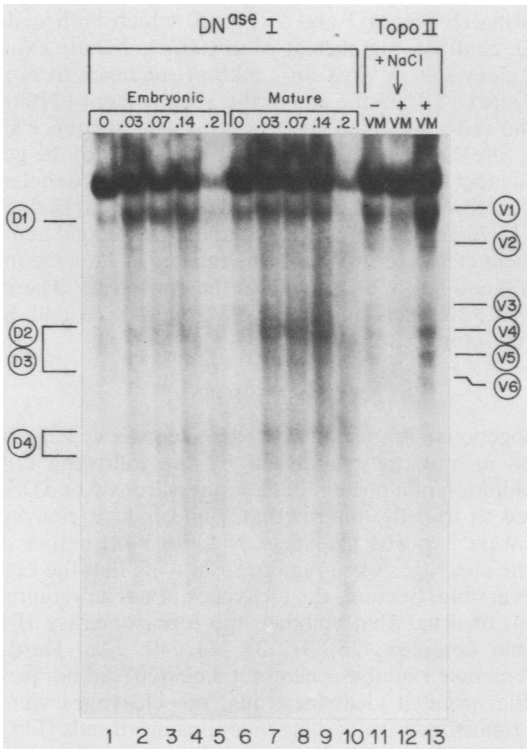


FIG. 5. DNase I hypersensitivity of the $\beta^A$-globin gene. Nuclei were prepared from either 14-day embryonic or adult erythrocytes and were suspended to $7.5 \times 10^7$/ml in buffer B. Lanes 1 to 5 contain embryonic nuclei and were digested with 0, 30, 70, 140, or 200 ng of DNase I (Sigma) for 12 min at 37°C, respectively. Lanes 6 to 10 contain adult nuclei digested with 0, 30, 70, 140, or 200 ng of DNase I (12 min at 37°C), respectively. Reaction experiments were terminated by adding EDTA to 0.5 M followed by ice quenching, adding detergent, and DNA purification (proteinase K digestion, phenol extraction, and ethanol precipitations). The ethanol precipitation was repeated, and after digestion with *Eco*RI, the digestion products were analyzed by Southern blotting. The in situ assay for topoisomerase II sites was performed as described in the legend to Fig. 2, except VM-26 was used as the inhibitor (30 μg/ml). Lane 11, nuclei without VM-26; lane 12, with VM-26, quenched with 0.5 M NaCl before the addition of detergent; lane 13, with VM-26. After lysis with detergent and DNA purification, the samples were digested with *Eco*RI. The Southern blot was hybridized with probe B (Fig. 4).

were similar if not identical. In this experiment, DNase I activity was titrated against the same number of nuclei from the two tissues under conditions that were optimal for DNase I (buffer B, see legend to Fig. 5). Under these conditions ($MgCl_2$, no ATP or drugs), we were unable to generate topoisomerase II-DNA covalent complexes; this was verified by performing immunoblot experiments across the step CsCl gradients to detect the presence of topoisomerase II (as in Fig. 1). A low level of endogenous nuclease is cutting at the 5′ site in both tissues (compare Fig. 5, lanes 1 and 6); however, it is clear that the intensity of hypersensitive bands increases after the exogenous addition of DNase I, which indicates that the hypersensitive sites detected by DNase I and the endogenous nuclease are identical. In addition, the major hypersensitive site we mapped in the $\beta^A$-globin gene chromatin in adult and embryonic nuclei is identical to the results published previously (22, 29). The cleavages which produced the various lower-molecular-weight bands (labeled D1 to D4) were not due to topoisomerase II since they were not inhibitor dependent. Furthermore, the addition of high salt (0.5 M) before lysis by sarcosyl did not reverse the cleavages. For comparison, topoisomerase II-induced cleavages were mapped in the same experiment, this time using VM-26 instead of *m*-AMSA (Fig. 5, lanes 11, 12, and 13). Parenthetically, the results of topoisomerase II mapping were the same for VM-26 and *m*-AMSA. In reaction experiments in which topoisomerase cleavages were mapped (lanes 11 to 13), assay conditions were different from those in reaction experiments containing DNase I (buffer B, lanes 1 to 10). It is clear that the topoisomerase II conditions (buffer B2 containing $CaCl_2$, see Materials and Methods) promote the activity of the endogenous nuclease (lane 11). Note, however, that the formation of lower-molecular-weight bands was significantly stimulated by VM-26 and was reversible by the addition of 0.5 M NaCl.

**In vitro cleavages of the $\beta^A$-globin gene with purified topoisomerase II.** Active chromatin structure around the $\beta^A$-globin gene in 12- to 14-day erythrocytes has been characterized by Felsenfeld and associates (13, 18, 22, 29). A nucleosome-free region contiguous to the 5′ hypersensitive site has been reported (29) as well as a 3′ enhancer (18) and in vivo footprints which identify sequences recognized by *trans*-acting factors (13, 14, 22). An in vitro analysis of topoisomerase II cleavage sites was carried out to catalog potential sites of catalytic activity. To improve resolution of cleavage products, we concentrated only on the 5′ half of the gene. A uniquely end-labeled fragment was prepared (from *Eco*RI at −820 to *Hin*dIII at +1060) (9) and was subjected to cleavages with purified chicken topoisomerase II (from adult erythrocytes; for details of the purification procedure, see Muller et al., Biochemistry, in press. The resulting cleavages were mapped by electrophoresis in native agarose gels so that only double-stranded cleavages were detected. The more prominent cleavages were cataloged along with several representative weaker sites (Fig. 6), and their positions and intensities are shown on the map in Fig. 7. An intense cleavage (site 2) was detected in the second intervening sequence of the $\beta^A$-globin gene, and weaker sites were detected at positions as marked.

**Comparison of hypersensitive sites and topoisomerase II sites.** The various sites in relationship to known features of the gene and its active chromatin structure are summarized (Fig. 7). We have attempted to assign cleavage sites with higher resolution than that attainable by Southern blotting (to single bases) by examining the sequence for homology to a topoisomerase II cleavage consensus sequence. A topoisomerase II consensus sequence which is applicable to type II enzymes from vertebrates was recently derived (42a). The sequence is as follows:

| −10 | −9 | −8 | −7 | −6 | −5 | −4 | −3 | −2 | −1 | +1 | +2 | +3 | +4 | +5 | +6 | +7 | +8 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 5′ A/G | N | T/C | N | N | C | N | N | G | T/C | N | G | G/T | T | N | T/C | N | T/C 3′ |

where N is any base, and cleavage by topoisomerase II occurs between −1 and +1. The strongest topoisomerase II cleavage site in vitro (site 2, Fig. 6 and 7) showed a 90% identity to the above consensus sequence, whereas other cleavages showed 70 to 80% matches to the consensus. The 5′ in vivo cleavage site (labeled A1, V1 in Fig. 7) matched the consensus at the 80% level, although this site was relatively weak in vitro (compared with a 90% match at site 2). The consensus sequence can predict sites in naked DNA; however, when the homology was in the 70 to 80% range, the intensity of cleavage was not always proportional to the degree of match to the consensus. This can be seen by

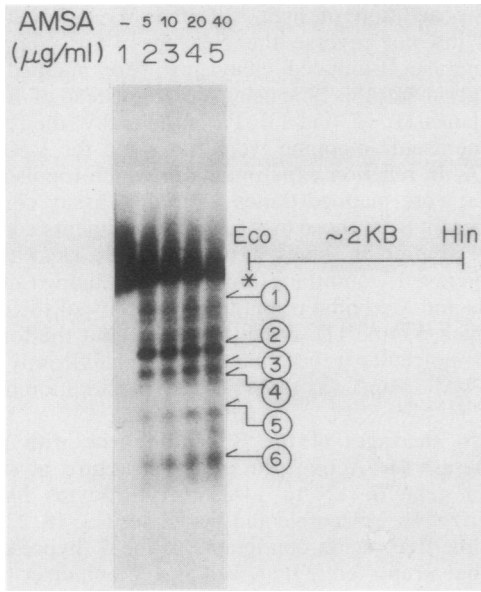


FIG. 6. In vitro cleavages of the 5′ end of the $\beta^A$-globin gene. The DNA fragment was uniquely end labeled at the *Eco*RI site that is 5′ of the $\beta^A$-globin gene to generate a DNA substrate extending from *Eco*RI (−820) to *Hin*dIII (+1060). Cleavage reaction experiments were carried out with purified chicken topoisomerase II (30 ng) and 10 ng of end-labeled fragment. Lanes: 1, the intact fragment (no topoisomerase or drugs) control; 2 through 5, fragment plus 5 to 40 μg of *m*-AMSA/ml as indicated.

comparing cleavages 3 and 5 (Fig. 6) which both match at 80%. In contrast, matches at 90 to 100% generally exhibited strong cleavages in vitro. In 2.8 kb of sequence from the 5′ *Eco*RI site (−820) to the end of the 3′ enhancer (+2100), two sites showed a 90% match to the consensus sequence shown above: Site 2 in the second intron and a site 3′ of the gene in the enhancer region (Fig. 7). The enhancer site (labeled V3, A2) aligned with topoisomerase II cleavages mapped in vivo with VM-26 and *m*-AMSA (Fig. 3 and 5). A second and more prominent cleavage site in the enhancer was detected in vivo (V4, A3) and has a 70% match to the consensus. The intron site (V2) was seen only with VM-26 in vivo and was a relatively minor site.

## DISCUSSION

Endogenous topoisomerase II cleavages have been mapped in situ, as established by the following criteria. First, addition of a protein denaturant (sarcosyl or SDS) was required to trap the intermediates and induce cleavage. If nuclei were exposed to excess NaCl (0.5 M) before detergent, the cleavages were repaired, showing that the reaction was reversible. Second, the cleavages in nuclei required the addition of drugs that stabilize the topoisomerase II-DNA cleavable complex (26, 31, 38, 41, 49, 61). Third, the topoisomerase I inhibitor camptothecin (20) did not produce a double-stranded cleavage; thus, the cleavages were not due to topoisomerase I acting on opposing strands (Fig. 4A). Fourth, it has been reported that $CaCl_2$ promotes trapping of the covalent topoisomerase II-DNA complex in vitro with purified topoisomerase II (34). $CaCl_2$ also stimulated cleavages in erythrocyte nuclei and enhanced the formation of the topoisomerase II-DNA covalent complexes; therefore, topoisomerase II is likely to be responsible for the double-

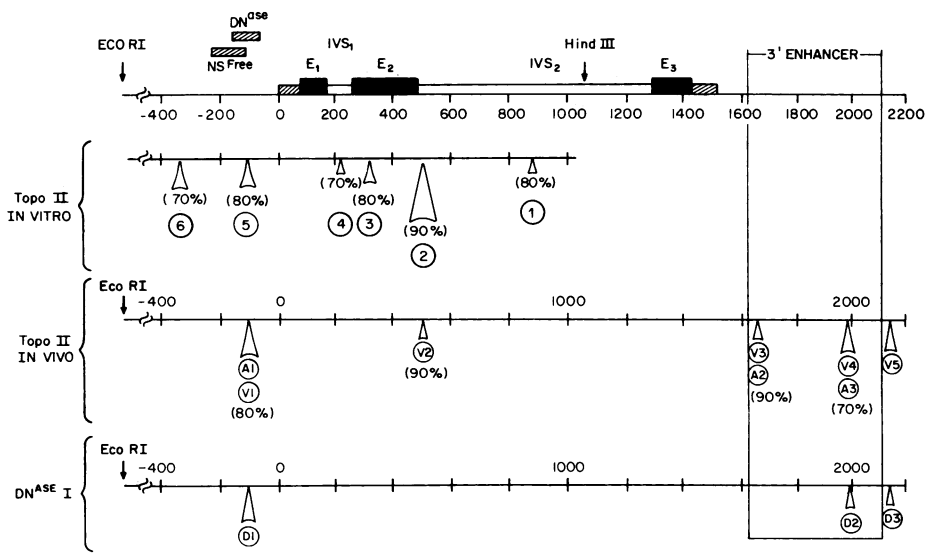


FIG. 7. Location of DNase I-hypersensitive sites and topoisomerase II sites in the $\beta^A$-globin gene. The top line shows the $\beta^A$-globin gene (leader sequence, introns [$IVS_1$, $IVS_2$], exons ($E_1$ to $E_3$), 3′ enhancer region) and features of active chromatin structure (DNase I-hypersensitive region and nucleosome-free regions are shown 5′ of the gene). 5′ And 3′ untranslated regions, ▨. The horizontal line immediately below the top diagram shows the topoisomerase II cleavages deduced in vitro. The circled numbers correspond to the numbered sites shown in Fig. 6 (only the sites between *Eco*RI and *Hin*dIII were mapped). The next horizontal line shows the positions of *m*-AMSA (A1 to A3)- and VM-26 (V1 to V5)-induced cleavages mapped in situ (Fig. 3 and 5). The bottom line is a display of DNase I-hypersensitive sites (D1 to D3) from Fig. 5. In all cases, the sizes of the open arrows below each map indicate relative strengths of the cleavages at particular sites; the values in parentheses correspond to the extent of the match to the topoisomerase II consensus sequence derived in this laboratory for vertebrate enzymes. Note that we have included in this summary only those sites for which sequence data are available (9, 18). Thus, some sites indicated in Fig. 5 (V6 and D4) are not shown.

stranded cleavages in nuclei. Fifth, the cleavage products were precipitated with SDS-K+ (45, 46). If the SDS-K+ precipitable DNA was not digested with proteinase K, its mobility was altered relative to free DNA, which further verifies the presence of a covalently linked polypeptide (data not shown). Sixth, the topoisomerase II sites mapped in nuclei aligned with sites that were homologous to a topoisomerase II consensus sequence that is applicable to vertebrate enzymes that have been tested (42a).

Previous studies have reported that endogenous topoisomerase II sites sometimes align closely with DNase I-hypersensitive regions in active chromatin (38, 61). These findings raised the interesting possibility that topoisomerase II catalytic sites are involved in some aspect of DNase I hypersensitivity, for example through the introduction of torsional stress in DNA (16, 23, 25, 39). The fact that not all topoisomerase II sites align with hypersensitive sites (Fig. 7) further suggests that topoisomerase II activity is unrelated to DNase I hypersensitivity; however, one cannot rule out the possibility that multiple topoisomerase II sites that flank the hypersensitive region could, either alone or in concert with other transcription factors (13, 14), collectively propagate DNase hypersensitivity. Our results argue against this because the 5′ DNase I-hypersensitive site maps identically in both embryonic and adult erythrocytes; however, there are no detectable topoisomerase II sites in the latter. Therefore, topoisomerase II activity and DNase I hypersensitivity appear as two independent phenomena, at least in the cell lineage in this study, since the altered chromatin state can persist in the absence of topoisomerase II activity in the gene region.

These results do not eliminate the possibility that topoisomerase II is necessary for active transcription of the β-globin gene in embryonic erythrocytes, possibly as related to the interplay between topoisomerase II and loop domains in the nucleus (2, 11, 15). Adult erythrocytes are not only transcriptionally inactive but also lack a nuclear matrix-associated loop domain structure found in most cell types (6, 33). The absence of DNA loops (6) and topoisomerase II cleavage sites in adult erythrocyte nuclei might indicate that these cells simply do not contain any cellular topoisomerase II; however, in the purification of topoisomerase II, we used adult erythrocytes as a starting source because large numbers of cells can easily be obtained and these cells are low in protease activity. Thus, the adult erythrocyte contains topoisomerase II activity (Muller et al., in press) although the levels are much lower than those in tissue culture cells (Muller and Mehta, unpublished data).

A tissue-specific factor conferring DNase hypersensitivity on the chicken $\beta^A$-globin gene has been identified and partially purified (13, 14). DNase I footprinting experiments have been carried out with the hypersensitive factor(s) and globin gene in vitro (14), and the results closely correlate with in vivo footprinting results (22). Our data indicate that topoisomerase II is also recognizing the 5′ region of the globin gene; given the extensive protection by the hypersensitive factor (−136 to −163 and −175 to −200) (22), we questioned whether topoisomerase II might not be excluded from this region, i.e., it seemed unlikely that topoisomerase II and the hypersensitive factor(s) would compete for identical sites. The 5′ endogenous topoisomerase II site in embryonic cells (site 5 in Fig. 7) maps approximately in between the two regions protected by the erythrocyte hypersensitivity factor (at −175) (14) on the basis of homology to the topoisomerase II consensus sequence. Thus, topoisomerase II and the hypersensitive factor(s) do not compete for the same sequence. Although speculative, we propose that topoisomerase II activity is detected at DNase-hypersensitive sites after they form, which is to suggest that DNase hypersensitivity is probably not initiated or maintained by topoisomerase II.

From this work, there is reasonable evidence to indicate that topoisomerase II and eucaryotic enhancers are functionally related. A major topoisomerase II recognition site was mapped in situ to the simian virus 40 regulatory region (61) near nucleotide 270, and on the basis of a match to the topoisomerase II consensus sequence (42a), the cleavage actually is between nucleotides 273 and 274. This places the site within the GTIIA region of the simian virus 40 enhancer as defined by the elegant studies of Chambon and associates (64). A search of published enhancer sequences in the GenBank and European Molecular Biology Laboratory data bases reveals that the topoisomerase II consensus sequence is enriched in these sequences compared with those of other sequences selected at random (42a). Finally, we demonstrate in the present work that endogenous topoisomerase II recognizes sites in the 3′ enhancer of the β-globin gene. The mapping data in situ (in simian virus 40 and the β-globin gene) strongly implicate topoisomerase II action with enhancer activity. At present, the in vivo functions of topoisomerase II might be linked to both transcription and DNA replication, because enhancers are probably important in both processes and because topoisomerase II catalytic sites are enriched in enhancers both in vivo and in vitro. Another common feature between replication and transcription is DNase hypersensitivity; as noted above, our data argue against an involvement of topoisomerase II in this aspect of chromatin structure. Additional experiments will be directed toward understanding the relationship between *cis*-active sequences and topoisomerase II.

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## LITERATURE CITED

1. **Bergsma, D. J., D. M. Olive, W. W. Hartzell, and K. N. Subramanian.** 1982. Territorial limits and functional anatomy of the simian virus 40 replication origin. Proc. Natl. Acad. Sci. USA **79**:381–385.
2. **Berrios, M., N. Osheroff, and P. Fisher.** 1985. *In situ* localization of DNA topoisomerase II, a major polypeptide component of the *Drosophila* nuclear matrix fraction. Proc. Natl. Acad. Sci. USA **82**:4142–4146.
3. **Brill, S. J., S. DiNardo, K. Voelkel-Meiman, and R. Sternglanz.** 1987. Need for DNA topoisomerase activity as a swivel for DNA replication and transcription of ribosomal RNA. Nature (London) **326**:414–416.
4. **Chandrasekharappa, S. C., and K. N. Subramanian.** 1987. Effects of position and orientation of the 72-base-pair-repeat transcriptional enhancer on replication from the simian virus 40 core origin. J. Virol. **61**:2973–2980.
5. **Chen, G. L., L. Yang, T. C. Rowe, B. D. Halligan, K. M. Tewey, and L. F. Liu.** 1984. Nonintercalative antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. J. Biol. Chem. **259**:13560–13566.
6. **Cook, P. R., and I. A. Brazell.** 1976. Conformational constraints on nuclear DNA. J. Cell. Sci. **22**:287–302.
7. **DeVillers, J., W. Schaffner, C. Tyndall, S. Lupton, and R.**

**Kamen.** 1984. Polyomavirus DNA replication requires an enhancer. Nature (London) **312**:242–246.
8. **DiNardo, S., K. Voelkel, and R. Sternglanz.** 1984. DNA topoisomerase II mutant of *Saccharomyces cerevisiae*: topoisomerase II is required for segregation of daughter molecules at termination of DNA replication. Proc. Natl. Acad. Sci. USA **81**: 2616–2620.
9. **Dolan, M., J. B. Dodgson, and J. D. Engel.** 1983. Analysis of the adult chicken β-globin gene. Nucleotide sequence of the locus, microheterogeneity at the 5′-end of β-globin mRNA, an aberrant nuclear RNA species. J. Biol. Chem. **258**:3983–3990.
10. **Duguet, M., C. Lavenot, F. Harper, G. Mirambeau, and A.-M. De Recondo.** 1983. DNA topoisomerases from rat liver: physiological variations. Nucleic Acids Res. **11**:1059–1075.
11. **Earnshaw, W. C., and M. S. Heck.** 1985. Localization of topoisomerase II in mitotic chromosomes. J. Cell Biol. **100**: 1716–1725.
12. **Eissenberg, J. C., I. L. Cartwright, G. H. Thomas, and S. C. R. Elgin.** 1985. Selected topics in chromatin structure. Annu. Rev. Genet. **19**:485–536.
13. **Emerson, B. M., and G. Felsenfeld.** 1984. Specific factor conferring nuclease hypersensitivity at the 5′ end of the chicken adult β-globin gene. Proc. Natl. Acad. Sci. USA **81**:95–99.
14. **Emerson, B. M., C. D. Lewis, and G. Felsenfeld.** 1985. Interaction of specific nuclear factors with the nuclease hypersensitive region of the chicken adult β-globin gene: nature of the binding domain. Cell **41**:21–30.
15. **Gasser, S. M., T. Laroche, J. Falquet, E. Boy de la Tour, and U. K. Laemmli.** 1986. Metaphase chromosome structure. Involvement of topoisomerase II. J. Mol. Biol. **188**:613–629.
16. **Gliken, G.C., I. Ruberti, and A. Worcel.** 1984. Chromatin assembly in *Xenopus* oocytes: *in vitro* studies. Cell **37**:33–41.
16a. **Heck, M. M. S., W. N. Hittelman, and W. C. Earnshaw.** 1988. Differential expression of DNA topoisomerases I and II during the eukaryotic cell cycle. Proc. Natl. Acad. Sci. USA **85**:1086–1090.
17. **Hertz, G. Z., and J. E. Mertz.** 1986. Bidirectional promoter elements of simian virus 40 are required for efficient replication of the viral DNA. Mol. Cell. Biol. **6**:3513–3522.
18. **Hesse, J. E., J. M. Nickol, M. R. Lieber, and G. Felsenfeld.** 1986. Regulated gene expression in transfected primary chicken erythrocytes. Proc. Natl. Acad. Sci. USA **83**:4312–4316.
19. **Holm, C., T. Goto, J. C. Wang, and D. Botstein.** 1985. DNA topoisomerase II is required at the time of mitosis in yeast. Cell **41**:553–563.
20. **Hsiang, H., R. Hertzberg, S. Hecht, and L. F. Liu.** 1985. Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. J. Biol. Chem. **260**:14873–14878.
21. **Jackson, D. A., and P. R. Cook.** 1986. A cell-cycle-dependent DNA polymerase activity that replicates intact DNA in chromatin. J. Mol. Biol. **192**:65–76.
22. **Jackson, P. D., and G. Felsenfeld.** 1985. A method for mapping intranuclear protein-DNA interactions and its application to a nuclease hypersensitive site. Proc. Natl. Acad. Sci. USA **82**: 2296–2300.
23. **Kmiec, E. B., and A. Worcel.** 1985. The positive transcription factor of the 5s RNA gene induces a 5S DNA-specific gyration in *Xenopus* oocyte extracts. Cell **41**:945–953.
24. **Li, J. J., K. W. C. Peden, R. A. F. Dixon, and T. Kelly.** 1986. Functional organization of the simian virus 40 origin of replication. Mol. Cell. Biol. **6**:1117–1128.
25. **Lilley, D. M.** 1983. Eucaryotic genes—are they under torsional stress? Nature (London) **305**:276–277.
26. **Liu, L. F., T. C. Rowe, L. Yang, K. M. Tewey, and G. L. Chen.** 1983. Cleavage of DNA by mammalian DNA topoisomerase II. J. Biol. Chem. **258**:15365–15370.
27. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Maxam, A. M., and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA **74**:560–564.
29. **McGhee, J. D., W. I. Wood, M. Dolan, J. D. Engel, and G. Felsenfeld.** 1981. A 200 base pair region at the 5′ end of the chicken adult β-globin gene is accessible to nuclease digestion. Cell **27**:45–55.
30. **Muller, M. T., W. P. Pfund, V. B. Mehta, and D. K. Trask.** 1985. Eucaryotic type I topoisomerase is enriched in the nucleolus and catalytically active on ribosomal DNA. EMBO J. **4**: 1237–1243.
31. **Nelson, E. M., K. M. Tewey, and L. F. Liu.** 1984. Mechanism of antitumor drug action: poisoning of mammalian DNA topoisomerase II on DNA by 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide. Proc. Natl. Acad. Sci. USA **81**:1361–1365.
32. **Nelson, W. G., L. F. Liu, and D. S. Coffey.** 1986. Newly replicated DNA is associated with DNA topoisomerase II in cultured rat prostatic adenocarcinoma cells. Nature (London) **322**:187–189.
33. **Newport, J. W., and D. J. Forbes.** 1987. The nucleus: structure, function and dynamics. Annu. Rev. Biochem. **56**:535–565.
34. **Osheroff, N., and E. L. Zechiedrich.** 1987. Calcium-promoted DNA cleavage by eucaryotic topoisomerase II: trapping the covalent enzyme-DNA complex in an active form. Biochemistry **26**:4303–4309.
35. **Palen, T. E., and T. R. Cech.** 1984. Chromatin structure at the replication origins and transcription initiation regions of the ribosomal genes of *Tetrahymena*. Cell **36**:933–942.
36. **Pederson, D. S., F. Thoma, and R. T. Simpson.** 1986. Core particle, fiber and transcriptionally active chromatin structure. Annu. Rev. Cell Biol. **2**:117–147.
37. **Rocha, E., J. R. Davie, K. E. van Holde, and H. Weintraub.** 1984. Differential salt fractionation of active and inactive genomic domains in chicken erythrocyte. J. Biol. Chem. **259**: 8558–8563.
38. **Rowe, T. C., J. C. Wang, and L. F. Liu.** 1986. In vivo localization of DNA topoisomerase II cleavage sites on *Drosophila* heat shock chromatin. Mol. Cell. Biol. **6**:985–992.
39. **Ryoji, M., and A. Worcel.** 1984. Chromatin assembly in *Xenopus* oocytes: *in vivo* studies. Cell **37**:21–32.
40. **Saavedra, R. A., and J. A. Huberman.** 1986. Both DNA topoisomerases I and II relax 2 μm plasmid DNA in living yeast cells. Cell **45**:65–70.
41. **Sander, M., and T. Hsieh.** 1983. Double strand DNA cleavage by type II DNA topoisomerase from *Drosophila melanogaster*. J. Biol. Chem. **258**:8421–8428.
42. **Shaw, J. L., J. Blanco, and G. C. Mueller.** 1975. A simple procedure for isolation of DNA, RNA and protein fractions from cultured animal cells. Anal. Biochem. **65**:125–131.
42a. **Spitzner, J., and M. Muller.** 1988. A consensus sequence for cleavage by vertebrate DNA topoisomerase II. Nucleic Acids Res. **16**:5533–5556.
43. **Stalder, J., A. Larsen, J. D. Engel, M. Dolan, M. Groudine, and H. Weintraub.** 1980. Tissue-specific DNA cleavages in the globin chromatin domain introduced by DNAase I. Cell **20**:451–460.
44. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
45. **Trask, D. K., J. D. DiDonato, and M. T. Muller.** 1984. Rapid detection and isolation of covalent DNA/protein complexes: application to topoisomerase I and II. EMBO J. **3**:671–676.
46. **Trask, D. K., and M. T. Muller.** 1983. Biochemical characterization of topoisomerase I purified from avian erythrocytes. Nucleic Acids Res. **11**:2779–2800.
47. **Uemura, T., H. Ohkura, Y. Adachi, K. Morino, K. Shiozaki, and Y. Yanagida.** 1987. DNA topoisomerase II is required for condensation and separation of mitotic chromosomes in *S. pombe*. Cell **50**:917–925.
48. **Udvardy, A., P. Schedl, M. Sander, and T. Hsieh.** 1986. Topoisomerase II cleavage in chromatin. J. Mol. Biol. **191**:231–236.
49. **Udvardy, A., P. Schedl, M. Sander, and T. Hsieh.** 1986. Novel partitioning of DNA cleavage sites for drosophila topoisomerase II. Cell **40**:933–941.
50. **Uemura, T., and M. Yanagida.** 1984. Isolation of type I and II DNA topoisomerase mutants from fission yeast: single and double mutants show different phenotypes in cell growth and

chromatin organization. EMBO J. **3**:1737–1744.
51. **Uemura, T., and M. Yanagida.** 1985. Mitotic spindle pulls but fails to separate chromosomes in type II DNA topoisomerase mutant: uncoordinated mitosis. EMBO J. **5**:1003–1010.
52. **Veldman, G., S. Lupton, and R. Kamen.** 1985. Polyomavirus enhancer contains multiple redundant sequence elements that activate both DNA replication and gene expression. Mol. Cell. Biol. **5**:649–658.
53. **Vosberg, H.-P.** 1985. DNA topoisomerases: enzymes that control DNA conformation. Curr. Top. Microbiol. Immunol. **114**: 19–101.
54. **Wang, J. C.** 1985. DNA topoisomerases. Annu. Rev. Biochem. **54**:665–697.
55. **Weintraub, H., and M. Groudine.** 1976. Chromosomal subunits in active genes have an altered conformation. Science **193**:848–856.
56. **Weintraub, H., A. Larsen, and M. Groudine.** 1981. Alphaglobin gene switching during the development of chicken embryos: expression and chromosome structure. Cell **24**:333–344.
57. **Wolffe, A. P., M. T. Andrews, E. Crawford, R. Lossa, and D. D. Brown.** 1987. Negative supercoiling is not required for 5S RNA transcription *in vitro*. Cell **49**:301–302.
58. **Wood, S. H., and J. M. Collins.** 1986. Preferential binding of DNA primase to the nuclear matrix in HeLa cells. J. Biol. Chem. **261**:7119–7122.
59. **Worcel, W.** 1987. Negative supercoiling is not required for 5S RNA transcription *in vitro*. A reply. Cell **49**:302–303.
60. **Wu, C., and W. Gilbert.** 1981. Tissue-specific exposure of chromatin structure at the 5′-terminus of the rat preproinsulin II gene. Proc. Natl. Acad. Sci. USA **78**:1577–1580.
61. **Yang, L., T. C. Rowe, E. M. Nelson, and L. F. Liu.** 1985. In vivo mapping of DNA topoisomerase II-specific cleavage sites on SV40 chromatin. Cell **41**:127–132.
62. **Yaniv, M., and S. Cereghini.** 1986. Structure of transcriptionally active chromatin. Crit. Rev. Biochem. **21**:1–26.
63. **Zenke, M., T. Grundstron, H. Matthes, M. Wintzerith, C. Schatz, A. Wilderman, and P. Chambon.** 1986. Multiple sequence motifs are involved in SV40 enhancer function. EMBO J. **5**:387–397.