Volume 11 Number 9 1983 Nucleic Acids Research

Biochemical characterization of topoisomerase I purified from avian erythrocytes

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Received 19 October 1982; Revised and Accepted 24 February, 1983

ABSTRACT

A type I topoisomerase has been purified from avian erythrocyte nuclei. The most pure fraction contains one major polypeptide of M_r = 105,000 (80% of total) and several minor ones of lower molecular weight. Active forms of the topoisomerase were identified by covalently binding the enzyme to ^{32}P-DNA, digesting with nuclease and detecting ^{32}P labeled peptides by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Topoisomerase activity, as measured by the ability to covalently bind DNA, is associated with the following peptides: M_r = 105, 83, 54 and 30,000. The similar chromatographic properties of the various forms of topoisomerase suggests a common structural identity as previously proposed for the HeLa topoisomerase I (Liu, L.F. and Miller, K.G. (1981) Proc. Natl. Acad. Sci. USA 78, 3487-3491). The avian enzyme is similar to other eucaryotic type I DNA topoisomerases in that it covalently binds double and single stranded DNA forming an enzyme linked to the 3'-phosphoryl end and after binding to single stranded DNA it can transfer the single stranded donor DNA to an acceptor DNA possessing 5'-OH end groups. The binding site size of topoisomerase on DNA has also been determined using micrococcal nuclease to digest unprotected DNA in the native enzyme/DNA complex. The enzyme blocks access to the helix over a span of 25 bp. These findings are discussed in light of the distribution and function of topoisomerase I in chromatin.

INTRODUCTION

The eucaryotic type I topoisomerase interconverts topological forms of circular DNA molecules (for recent reviews see 8, 15, 37). The enzyme was originally discovered in nuclear extracts of mouse embryos (9), and is apparently a ubiquitous nuclear protein having been identified in human cell lines (17, 19), calf thymus (28, 29), rat liver (10), and avian erythrocytes (4).

Topoisomerase I relaxes supertwisted DNA by introducing a single strand interruption in the sugar phosphate backbone of the DNA substrate and then reseals the free DNA ends (for reviews see 8, 15). This mechanism clearly distinguishes the type I enzymes, which change the linking number in steps of one (30) from the type II enzymes which catalyze the breaking and resealing

of both strands of the helix and change the linking number in steps of two (5).

Recent studies on the mechanism of topoisomerase catalyzed reactions have shown that a covalent DNA/enzyme intermediate is formed (7). A covalent complex was predicted in the early studies with the procaryotic equivalent of this enzyme (the Ω protein) to explain how the nicking and resealing of the phosphodiester bond in DNA could be accomplished without an energy cofactor (36). Recently, type I enzymes have been shown to catalyze a number of different reactions in addition to relaxation of supertwisted DNA. The type I enzymes, for example, efficiently catenate open circular DNA (6). Type I enzymes have also been shown to promote the covalent transfer of single stranded donor DNA to heterologous acceptor DNA (2, 17). Binding of the enzyme to single stranded DNA results in cleavage of that DNA, and the spontaneous formation of a covalent DNA/enzyme intermediate in the absence of protein denaturants. In this arrested state, the enzyme retains its ability to complete the catalytic cycle of rejoining the single stranded DNA fragment to the end of an acceptor DNA. These studies have provided direct proof that single strand breaks and covalent DNA/topoisomerase complexes are functional intermediates in the reaction (2, 17).

While these interesting studies establish the fundamental reaction mechanism of topoisomerase I, the physiological function of the enzyme remains obscure. In vivo, eucaryotic DNA in nucleochromatin is organized into domains of negatively supercoiled loops containing on the average 400 nucleosomes per loop (3). Supercoiling is evidently due to nucleosomal structure (16) such that most superhelical turns of the DNA are confined to nucleosomes while internucleosomal DNA is in general torsionally unrestrained (35). Enzymes that transiently break and rejoin DNA may be responsible for topological interconversion of circular chromatin segments during DNA repair, replication, transcription and possibly genetic rearrangements. In support of this view are the observations that replicating and transcriptionally active chromatin are structurally distinct from bulk chromatin (27, 38). Topoisomerases may modulate or adjust DNA topology in the replication or construction of transcriptionally active chromatin in a fashion similar to the combined action of topoisomerase I and nucleosome assembly factors in vitro (21).

As a first step toward establishing a tractable system for investigating the role of topoisomerase I in chromatin structure and function, we have purified the enzyme from avian cells. The avian system has the advantage of

providing a convenient source for purification of relatively large quantities of the enzyme using avian erythrocytes. In addition, the availability of transformed avian cell lines provides the opportunity to study the same enzyme in a transcriptionally active genetic background.

EXPERIMENTAL PROCEDURES

Conditions for Detection of Covalent Binding of Topoisomerase to DNA. Exposure of protein-SDS complexes to K^+ leads to the formation of an insoluble potassium-SDS-protein complex. Since SDS does not bind to double stranded nucleic acids, treatment of a mixture of protein and DNA with SDS/KCl affords a rapid method for selectively precipitating proteins leaving free nucleic acids in the supernatant. Therefore, a protein which covalently binds to DNA results in the precipitation of that DNA using the SDS-K^+ method. (Additional details of this stoichiometric assay for topoisomerase will be published elsewhere.) Nick translated DNA and the purified topoisomerase were routinely assembled in a reaction volume of 50 ul. The reactions were incubated at 30° for 60 min in low salt binding buffer (42 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5 mM PMSF) and terminated by the addition of 1 ml of a solution of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 ug BSA/ml, 20 ug calf thymus DNA/ml, and 1% SDS. The reaction mixture was vortexed, 0.3 ml of 2.5 M KCl added, vortexed again and incubated on ice for 30 min. The resulting SDS-K^+ precipitates were collected by centrifugation (1000 g for 5 min), resuspended in 1 ml of TKE buffer (10 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM EDTA) and vortexed. After a second wash with TKE buffer, the final precipitate was resuspended in 0.5 ml of 1 N HCl, vortexed to dissolve the precipitate, 0.5 ml of 1 N NaOH added, and radioactivity measured.

Purification of Topoisomerase I. Nuclei were prepared from 1.5 liters of chicken blood using the following method (all operations performed at 4°). Heparinized blood was stored frozen (at -70°) and quickly thawed as needed. An equal volume of Tris buffered saline (TBS, 10 mM Tris-HCl (pH 7.85), 150 mM NaCl) was added and the insoluble clots removed by filtration through sterilized cheese cloth. RBC's were removed from suspension by centrifugation (5,000 g x 10 minutes) and the resulting pellet resuspended in 2.5 liters TBS. Filtering and centrifugation steps were repeated and the pellet resuspended in 2.4 liters TEMP buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 4 mM $MgCl_2$, 0.5 mM PMSF), and the RBC's centrifuged at 6,000 g for 10 minutes in a Sorvall GS-3 rotor. This pellet was resuspended in 2.4 liters of TEMP, and the centrifugation step was repeated. Most of the hemoglobin

and cytosol proteins were washed out in this final centrifugation step yielding a pellet of isolated nuclei (2.9 x 10^{12} total nuclei). The nuclear pellet was resuspended in buffer A (50 mM Tris-HCl (pH 7.55), 1 mM EDTA, 0.5 mM PMSF, 10% glycerol, 50 mM sodium bisulfite) at a concentration of 2.9 x 10^{9} nuclei/ml (total volume of 1 liter).

Solid NaCl was added with stirring to a final concentration of 0.4 M and the extraction continued for 1 hour at 0^{o}. An additional 500 ml of buffer A containing 0.4 M NaCl was added and polymin-P added with rapid stirring to a final concentration of 0.75%. The polymin-P was prepared as a 10% (v/v) stock solution as described by Jendrisak and Burgess (18). Stirring was continued for 15 minutes and the suspension centrifuged (10,000 g x 20 minutes). The volume of the supernatant was measured (1250 ml), and solid ammonium sulfate was slowly added to 257 g/l with stirring. Stirring was continued for 30 minutes and the suspension centrifuged (10,000 g x 20 minutes), and the pellet stored at -70^{o}. An additional 120 g/l of solid ammonium sulfate was slowly added with stirring and centrifugation repeated. Solid ammonium sulfate (132 g/l) was then added to the supernatant and the centrifugation step repeated. The final pellet was carefully drained and the sides of the tube wiped clean with autoclaved tissue paper. The pellet was then resuspended in 25 ml of buffer A and diluted with buffer A to give a final concentration of 0.2 M ammonium sulfate (loading buffer) as measured by conductivity. The final volume at this stage was approximately 50 ml.

This extract was applied to a 1.5 x 27 cm column of phosphocellulose pre-equilibrated with the loading buffer. The void volume was collected and the column washed with 3-4 column volumes of 0.2 M ammonium sulfate in buffer A (flow rate 16 ml/hour). Proteins were eluted with a 500 ml linear gradient of 0.2-0.8 M ammonium sulfate in buffer A (flow rate of 13 ml/hour) and 2.5 ml fractions were collected. Fractions were assayed for topoisomerase, and active fractions were pooled.

A 1.5 x 16.4 cm column of phenyl-Sepharose was pre-washed with 3 column volumes of distilled water containing 50% (v/v) glycerol and then equilibrated with buffer A containing 0.8 M ammonium sulfate. The pooled fraction was then adjusted to 0.8 M ammonium sulfate and the fraction loaded at a flow rate of 13 ml/hour. The topoisomerase was eluted with a 250 ml linear gradient of 0.8-0 M ammonium sulfate in buffer A, and 2 ml fractions were collected. The peak of activity was localized, and only those fractions showing maximum topoisomerase activity were pooled. The pooled fraction was stored at -70^{o} in 50 ul aliquots.

Freezing and thawing of the purified enzyme resulted in a detectable decrease in activity; for this reason all experiments were carried out using aliquots that had not been subjected to a freeze/thaw cycle. Dialysis of the pure enzyme was also avoided (see text). The loss of activity with freezing/thawing or dialysis was eliminated by dilution of the enzyme with a stabilization buffer of 80% glycerol, 50 ug BSA/ml, 0.2 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM PMSF, and 1 mM EDTA.

Other Procedures. Topoisomerase activity was measured by relaxation of supercoiled plasmid DNA (22). DNA was either end labeled (24) or nick translated (33) as indicated. DNA of 160 bp was purified from chromatosomes (39). SDS-polyacrylamide electrophoresis was performed as described by Laemmli (20).

RESULTS

Presence of Topoisomerase I in MSB-1 and Erythrocyte Nuclei. In preliminary experiments, we determined that an ATP, Mg^{++} independent topoisomerase was present in salt extracts of chicken erythrocyte nuclei and in nuclei isolated from the continuous avian cell line, MSB-1 cells (1). Figure 1 shows the relative level of topoisomerase activity present in 0.4 M salt extracts of nuclei from these two sources. The level of activity on a per weight basis of protein and a per cell basis is greater in the transformed cell line. A mixing experiment (Figure 1, Lane D) demonstrates that interfering material in the RBC extract is not responsible for the diminished levels of activity in this case. These results show that less topoisomerase activity is present in terminally differentiated RBC nuclei than the MSB-1 nuclei; however, we cannot exclude the possibility that in either case the recovered activity is not representative of the total, since differential recovery of the enzyme has been reported from growing versus nongrowing cells (25). Although RBC's appear to contain less extractable topoisomerase/cell, the availability of large quantities of chicken blood provides an extremely economical source of the enzyme without resorting to expensive large scale tissue culture.

Purification of Topoisomerase I from RBC Nuclei. The topoisomerase is efficiently (>90%) extracted from isolated erythrocyte nuclei by treatment with 0.4 M NaCl at 0^{o} for 1 hour. After centrifugation, the nuclear residue contains the bulk of the inner histones and DNA. The supernatant fraction, containing the topoisomerase, constitutes fraction I. Contaminating nucleic acids in fraction I were removed by precipitation with the polycation, polymin-P. Preliminary experiments demonstrated that polymin-P in the range

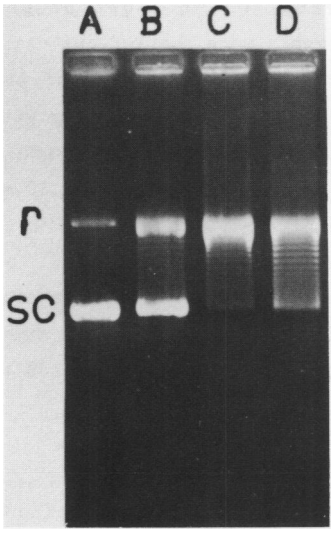

Figure 1. Levels of Topoisomerase I Activity in RBC and MSB-1 Nuclear Extracts. Nuclei were isolated from MSB-1 cells or RBC's and washed with TEMP buffer to remove cytosol proteins. The nuclear pellet was resuspended to 6 x 10^8 nuclei/ml (RBC nuclei) or 7 x 10^7 nuclei/ml (MSB-1 nuclei) and extracted with 0.4 M NaCl as described in methods. The total protein concentrations were 0.42 mg/ml and 0.25 mg/ml for RBC and MSB-1 nuclei, respectively. Topoisomerase activity was assayed by relaxation of form I DNA. Lane A: Reference DNA; SC = supercoiled DNA; r = relaxed DNA. Lane B: DNA was treated with 0.84 ug of the RBC nuclear extract. Lane C: The DNA was treated with 0.5 ug of the MSB-1 nuclear extract. Lane D: incubation with 0.84 ug plus 0.5 ug of RBC and MSB extract, respectively.

of 0.75 to 1% effectively precipitated nucleic acids and presumably some acidic proteins (18), leaving the topoisomerase I in the supernatant fraction. Topoisomerase activity was not detectable upon back extraction of the 1% polymin-P precipitate with 0.4 M NaCl. A final concentration of 0.75% polymin-P was chosen since a higher specific activity topoisomerase fraction was obtained; at higher polymin-P concentrations (up to 2%) the majority of the enzyme was precipitated.

Residual polymin-P in the topoisomerase active supernatant must be removed since the polycation strongly interferes with the assay and subsequent column chromatography steps. The residual polycation is conveniently removed by ammonium sulfate precipitation. The activity did not precipitate in 33%-45%, 45%-55%, or 55%-65% of saturation; however, the 85% saturation cut contained the precipitated topoisomerase and no interfering polymin-P. Therefore, protein precipitating below 65% saturation was discarded and the concentration of ammonium sulfate increased to 85% saturation, followed by centrifugation to collect the precipitate. The protein was then resuspended in buffer A in approximately 1/70 of the original volume (=fraction II) prior to application to a phosphocellulose column).

The topoisomerase activity is quantitatively bound to phosphocellulose in 0.2 M $(NH_4)_2SO_4$ in buffer A. The column flow through and wash contained no detectable activity. The peak of topoisomerase activity was eluted in the

range of 0.35-0.4 M $(NH_4)_2SO_4$ and contained approximately 2% of the protein applied to the column.

Optimal conditions for topoisomerase binding to phenyl-Sepharose were found to be 0.8 M $(NH_4)_2SO_4$ in buffer A. Accordingly, the phosphocellulose pool (fraction III) was adjusted by addition of solid ammonium sulfate, to 0.8 M, prior to chromatography on phenyl-Sepharose. The activity was eluted with a decreasing linear salt gradient (0.8 M to 0.0 M $(NH_4)_2SO_4$ in buffer A). The peak of activity was localized in the range of 0.4 to 0.5 M salt. This fraction (fraction IV containing 0.17 mg protein) was stored in small aliquots at -70°. The activity was stable after a single freeze/thaw cycle, but decreased variably with repeated freezing and thawing. More recently, we found that a five- to ten-fold dilution of this fraction into 80% glycerol, 50 ug/ml BSA, 0.2 M NaCl, 1 mM PMSF, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, stabilized the activity. Dialysis of the most pure fraction was, in all cases tested, attended by a decrease in total activity and the appearance of lower molecular weight peptides (data not shown).

SDS-polyacrylamide gel electrophoresis was carried out on protein fractions at various stages in the purification (Figure 2). The pure fraction (phenyl-Sepharose pool) contains two predominant polypeptides of M_r = 105 and 83,000 and a faint band of M_r = 87,000. Microdensitometry and integration of peak intensity reveals that the largest polypeptide represents 70%-80% of the total protein in this fraction. The relative amounts of the lower molecular weight polypeptides were somewhat variable from one preparation to the next (repeated 6 times); however, in all topoisomerase active fractions after the crude nuclear extract (fraction I), the M_r = 105,000 peptide band was present. Since this peptide co-purifies with the topoisomerase I activity, it is likely to be the denatured form of the enzyme. This molecular weight is in close agreement with recent reports on the HeLa (22) and wheat germ topoisomerases (13). Although the molecular weight of topoisomerase I was originally reported to be in the range of 60,000 to 70,000 (10, 19), more recent studies suggest that the M_r = 60,000 to 70,000 topoisomerase are cleavage products of the 100,000 molecular weight forms (22).

The purification procedure is summarized in Table 1. Due to the semiquantitative nature of the topoisomerase agarose gel assay and the fact that the level of activity is dramatically influenced by interfering proteins in the crude fractions, we did not use specific activity as a measure of purity. Instead, the absolute amount of the M_r = 105,000 polypeptide was

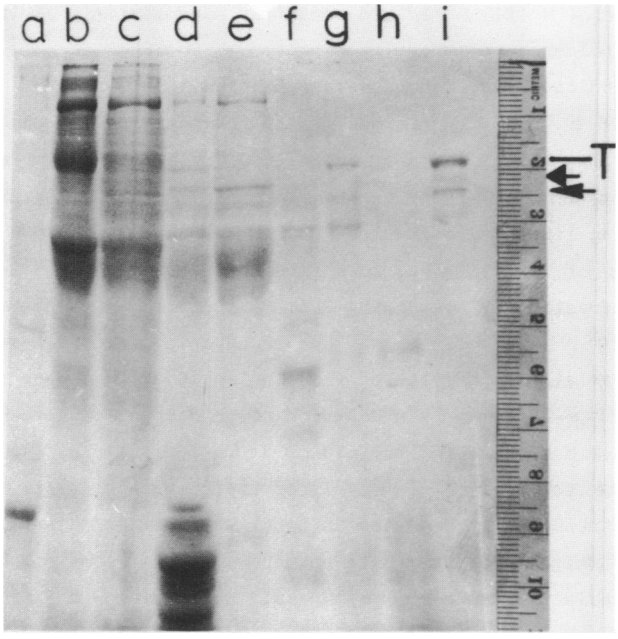

Figure 2. SDS-Polyacrylamide Gel Electrophoresis of Various Fractions. The various protein fractions were boiled in SDS sample buffer and loaded on a 7.5% SDS-polyacrylamide slab gel. Molecular weights were determined by comparison with markers (myosin, 200,000; β-galactosidase, 116,000; phosphorylase B, 92,500; bovine serum albumin, 68,000; ovalbumin, 44,000; carbonic anhydrase, 31,000). Lane A = 0.4 M NaCl nuclear extract; Lanes B,C,D = 45%, 65%, and 85% ammonium sulfate pools, respectively; Lane E = phosphocellulose column flow through; Lane F = phosphocellulose column wash; Lane G = phosphocellulose active pool; Lane H = phenyl-Sepharose column flow through; Lane I = phenyl-Sepharose active pool. The amount of protein loaded on the gel was determined for those fractions which contained topoisomerase activity: Lane A, 80 ug; Lane D, 210 ug; Lane G, 15 ug; Lane I, 3.5 ug. The M_r = 105,000 band is marked with a 'T' and two less intense bands with arrows (M_r = 87 and 83,000).

determined in fractions II-IV by analysis of peak intensity of a scanned gel. This approach is less biased than enzymatic determinations of yields since multiple molecular weight forms of the avian topoisomerase exist which are catalytically active (22, see Figure 4 below).

The Avian Topoisomerase Covalently Binds to DNA. The purified enzyme was denatured by 0.5% SDS after incubation with native ^{3}H -λ DNA (ratio of 4,000 enzyme molecules/DNA molecule) and the products analyzed by equilibrium density gradient centrifugation in CsCl at neutral pH. Figure 3 shows that denaturation of the enzyme in the presence of DNA results in a complex which

Table I. Purification Summary of the M_r = 105,000 Topoisomerase

Purification Step	Volume (ml)	Total Protein (mg)	M_r=105,000 Protein (mg)[a]	% M_r=105,000 Protein of Total Protein	Fold Purification	Yield (%)
I. Nuclear extract[b]	1300	1586	--	--	--	--
II. 85% ammonium sulfate	25	363	0.612	0.17	1	100
III. Phosphocellulose pool	75	8.4	0.378	4.5	26.5	61
IV. Phenyl-Sepharose pool	8	0.168	0.140	83.3	487	22

[a] The M_r = 105,000 protein was identified as topoisomerase I (see text). The concentration of this protein was determined by quantitative microdensitometry of a Coomassie blue stained gel using a LKB soft laser densitometer interfaced with an Apple computer. A program was used to integrate the area of the M_r = 105,000 protein peak and the absolute amount of protein determined by correlating known amounts of the purified topoisomerase with peak intensity.

[b] The M_r = 105,000 protein could not be unambiguously identified in the nuclear salt extract by SDS-polyacrylamide gel electrophoresis; therefore, the extent of purification (fold purification) is actually underestimated. From enzymatic determinations, we routinely obtain a four to five fold purification by ammonium sulfate precipitations; thus, we estimate that the enzyme has been purified about 2,500 fold.

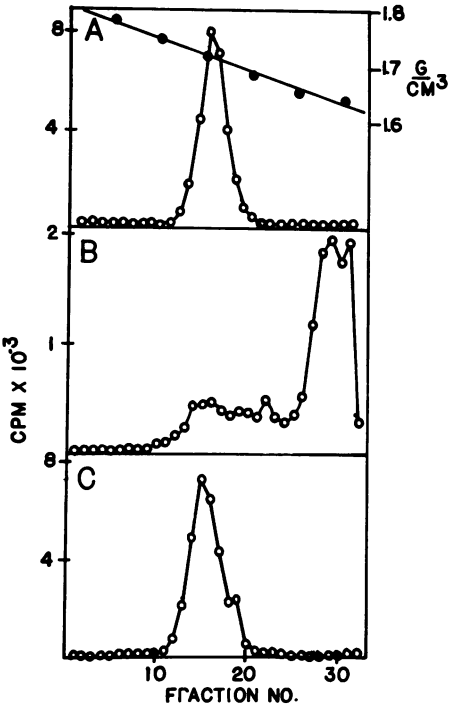

Figure 3. CsCl Equilibrium Gradient Analysis of the DNA/Topoisomerase Complex. The purified topoisomerase (36 ng) and nick translated λ DNA (20 ng) were incubated in a final volume of 25 ul in low salt binding buffer as described in "Experimental Procedures." The reactions were terminated after 30 minutes by the addition of 0.5% SDS and centrifuged to equilibrium in neutral CsCl (SW 50.1, 26,000 RPM for 60 h). (A) λ DNA marker; (B) λ DNA incubated with topoisomerase, treated with 0.5% SDS; (C) as in (B) but the reaction products were digested with proteinase K (800 ug/ml, 30 minutes at 37°) prior to centrifugation.

exhibits a lower buoyant density in CsCl. Treatment of the reaction products with proteinase K prior to centrifugation in CsCl resulted in labeled DNA at the expected density of pure λ DNA (Figure 3C), showing that protein is directly responsible for the decreased buoyant density. The fact that the trapped DNA/topoisomerase intermediate resists dissociation by high salt (>5 M, Figure 3B) extremes in pH (<3.5 and >12.5), boiling, 8 M urea and 4 M guanidine hydrochloride (data not shown) argues that the protein is covalently coupled to the DNA substrate.

Identification of Multiple Forms of Topoisomerase. The two prominent polypeptides in fraction IV displayed very similar chromatographic properties since we were unable to separate them on phosphocellulose, hydroxyapatite, phenyl-Sepharose and blue Sepharose columns (not shown). One explanation for these observations is that the smaller peptide is structurally related to the M_r = 105,000 peptide and results from proteolysis of the higher molecular weight form (22). In support of this, we observed that dialysis of fraction IV or incubation at 37° resulted in an apparant increase in intensity of the M_r = 83,000 peptide and a corresponding decrease in the M_r = 105,000 peptide band (data not shown). The loss of the 105,000 dalton peptide was also

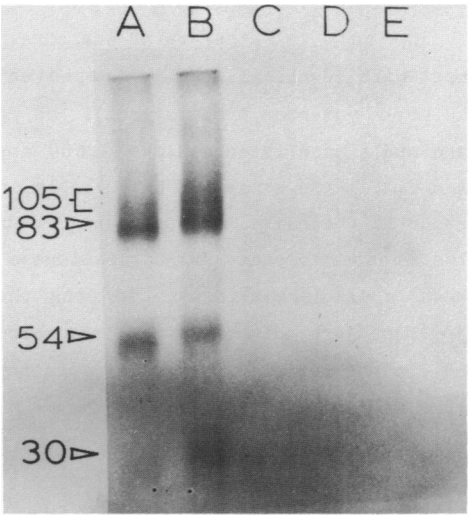

Figure 4. Analysis of Multiple Forms of Topoisomerase by Cross Linking to ^{32}P λ-DNA. Reactions (0.1 ml final volume) containing 2 ug topoisomerase/ml, 1 ug/ml nick translated ^{32}P labeled pBR-322 DNA (2 x 10^7 dpm/ug), 50 ug BSA/ml were incubated for 30 min at 30° in low salt binding buffer. The reactions were terminated by the addition of 0.1 volume of 0.2 M sodium acetate (pH 3.65) to denature the enzyme and cross link it to the DNA substrate (7). The reactions were placed on ice for 10 min and a pretitrated amount of 1 M Tris-HCl (pH 7.8) added to give a final pH of 7.4. $MgCl_2$ and PMSF were added to 10 mM and 1 mM respectively, followed by 25 ug DNase I/ml and the reactions incubated for 15 min at 37°. Proteins were precipitated with 25% trichloroacetic acid and analyzed on a 7.5% SDS polyacrylamide slab gel with molecular weight standards described in Figure 2. The gel was dried and exposed to Kodak XAR-5 film with an Ilford intensifying screen for 24 h. Longer exposures did not reveal any additional bands. Lane A: Fraction III topoisomerase; Lane B: Fraction IV topoisomerase; Lanes C and D as in A and B respectively except the low salt binding buffer was supplemented with 0.5 M NaCl; Lane E: Control, without topoisomerase.

attended by a variable but not complete decrease in activity of the enzyme. To identify directly which of the peptides has topoisomerase activity, we used a modified procedure described by Rekosh et al. (32). Briefly, the enzyme was covalently bound to ^{32}P DNA (7) and the DNA/topo complex digested with deoxyribonuclease I. The digests were then analyzed on SDS-polyacrylamide gels to identify peptides labeled with ^{32}P. Figure 4 shows the result of such a cross linking experiment using partially purified topoisomerase from fraction III and the purified enzyme from fraction IV. Four labeled bands were detected with the following molecular masses: 105, 83, 54, and 30 kilodaltons. Fractions III (phosphocellulose) and IV

(phenyl-Sepharose) yielded identical results (Figure 4, Lanes A and B). While the M_r = 105,000 band is rather faint and somewhat diffuse, we have repeated this experiment with identical results (repeated 4 times and using different purified topoisomerase preparations). We repeatedly observed a faint M_r = 105,000 band and a more intense M_r = 83,000 and 54,000; however, the M_r = 30,000 band was somewhat variable in intensity from one topoisomerase preparation to the next. The following controls support the idea that the labeled bands represent DNA/topoisomerase complexes: (1) the bands do not appear when topoisomerase is omitted from the reaction (Figure 4, Lane E); (2) the bands are not observed if the topoisomerase binding to DNA is carried out under conditions where the enzyme cannot efficiently bind to the helix (in 0.5 M NaCl, see Lanes C and D, Figure 4) (25); (3) the ^{32}P-labeled bands are proteinase K sensitive (data not shown); (4) after cross linking, if the nuclease digestion is omitted, a high molecular weight complex is observed which fails to enter the gel (data not shown); (5) labeled bands are not observed if the low pH cross linking step is omitted (not shown).

Characterization of the Purified Topoisomerase. Incubation of the purified enzyme in 100 mM NaCl with a 10-fold molar excess of form I pBR-322 DNA results in the complete relaxation of the DNA in a 60 minute incubation at 30°. We conclude, therefore, that the avian topoisomerase acts catalytically. The purified enzyme does not contain detectable levels of contaminating nucleases since incubation with form I pBR-322 DNA in the presence of 10 mM $MgCl_2$, 50 mM NaCl did not result in the formation of form II (open circular) or form III (linear) DNA species (data not shown). The activity of the enzyme is not significantly influenced by 0.5% NP-40 or Triton-X 100; however, low levels (0.2%) of SDS completely denature the topoisomerase. The enzyme displays a rather broad monovalent salt optimum of 50 to 200 mM NaCl; at NaCl concentrations in excess of about 0.4 M the enzyme cannot bind to the helix (25) (see Figure 4). The relaxation of supercoiled DNA is not significantly influenced by the addition of $MgCl_2$ (tested over range of 1 to 20 mM); however covalent binding of the enzyme to DNA is stimulated by 5 mM Mg^{++} (Muller and Trask, submitted for publication). ATP is not required for activity of the purified enzyme.

These observations suggest that this topoisomerase is a type I enzyme. This was verified by the following experiment. Supercoiled pBR-322 DNA was incubated with purified topoisomerase at 20°C and subjected to preparative electrophoresis in an agarose gel at 4°C. Under these conditions we resolved

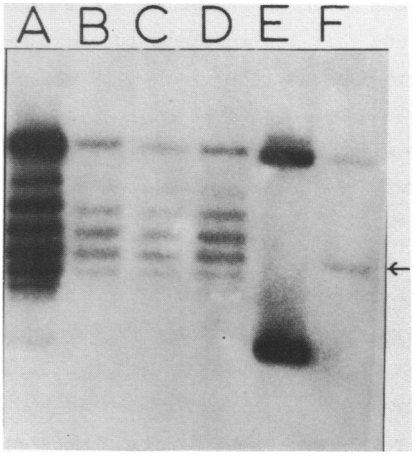

Figure 5. The avian topoisomerase changes the DNA linking number in steps of one. Plasmid DNA (pBR 322, 100 ug/ml) was incubated with 125 ug topoisomerase/ml for 2 hours at 20° in 100 mM ammonium chloride, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.9). The reaction was stopped with the addition of SDS to 1%, treated with 50 ug proteinase K/ml for 30 min at 56°, and precipitated with 2 volumes of cold ethanol. The DNA was subjected to preparative electrophoresis in a 0.8% agarose gel at 4°, after which a 3 mm vertical segment of the DNA containing lane was stained with ethidium bromide. The region of the gel not exposed to ethidium bromide, containing DNA with a unique linking number was carefully sliced out and electroeluted. Following ethanol precipitation, DNA with a unique linking number was resuspended in 10 mM Tris-HCl (pH 7.8), 1 mM EDTA and incubated with topoisomerase. All reactions (final volume of 25 ul) were carried out in 100 mM ammonium chloride, 1 mM EDTA, 10 mM Tris-HCl (pH 7.9), and 125 ug/ml avian topoisomerase at 20°. The reactions were terminated with a one-tenth volume of 10% SDS and incubated at 56° with 50 ug proteinase K/ml, prior to electrophoresis in a 0.8% agarose gel at 2 V/cm at 4°. The DNA was transferred to nitrocellulose by the method of Southern (34), hybridized, and exposed to Kodak XAR-5 film for 6 hours. Lane A, 200 ng of form I DNA incubated at 20° for 2 hours with 3 units of calf thymus topoisomerase I using manufacturer's specifications. Lane B, 200 ng of form I DNA incubated with purified avian topoisomerase for 2 hours. Lane C, the isolated topoisomer incubated with avian topoisomerase for 1 hour. Lane D, the topoisomer incubated with avian topoisomerase for 2 hours. Lane E, 300 ng of form I and form II pBR 322 DNA. Lane F, the topoisomer incubated without topoisomerase. Approximately 1/3 less total DNA was added to Lane F than in Lanes C and D.

the expected gaussian distribution of topoisomers. A unique topoisomer was excised (Figure 5, Lane F, arrow), purified, and then again incubated with purified avian topoisomerase under the same conditions. The products of this reaction were again analyzed by agarose gel electrophoresis. As shown in Figure 5, Lanes C and D, a full gaussian distribution of topoisomers was

regenerated. Since the linking number of the topoisomerase was modified in steps of one, we conclude that the topoisomerase purified from avian erythrocytes is a type I enzyme (30). In addition, the purified enzyme will catenate open circular DNA but not covalently closed circular DNA (unpublished observation) (6). Catenanes were demonstrated by electron microscopy and the formation of high molecular weight pBR-322 DNA complexes; the latter were released by treatment with Eco RI digestion to form linear DNA.

The purified topoisomerase (fraction IV) is probably contaminated with low levels of protease since dialysis overnight against buffer A or incubation at 37° for 4 hours (with and without 0.1% SDS) results in a decrease in intensity of the M_r = 105,000 polypeptide and a corresponding increase in the M_r = 83,000 form (not shown). The conversion of the higher to the lower molecular weight form was not prevented by 1 mM PMSF, 50 mM sodium bisulfite, or a combination of the two. Addition of carrier BSA (50 ug per ml) to the purified enzyme did, however, minimize the conversion.

The Avian Topoisomerase is Cross Linked to 3' Ends. When the nicking and closing cycle of the topoisomerase is arrested by protein denaturation, a DNA/enzyme complex is formed bearing the enzyme covalently coupled to the 3' phosphoryl end of DNA at the site of the nick (7, 17). We have developed a stoichiometric enzyme-DNA cross linking assay (Muller and Trask, submitted for publication) to determine ir the avian enzyme also displays the property of the 3' end association. When the enzyme is incubated with either single or double stranded DNA followed by denaturation with SDS, a covalent complex is formed. Those DNA molecules which contain a covalently coupled topoisomerase can be separated from free DNA by the addition of KCl. An insoluble SDS-K^+ complex forms which contains free protein and DNA-protein intermediates. The SDS-K^+ precipitate is then washed and the amount of radioactive DNA determined (details of this stoichiometric assay for topoisomerase to be published elsewhere). Since free DNA does not bind SDS, it is not detected in the SDS-K^+ precipitate. That the method accurately measures the covalent binding of topoisomerase to DNA was verified by comparing the SDS-K^+ assay with the fraction of λ DNA shifted to a lower buoyant density in CsCl. The fraction of λ DNA of the lower density in the experiments shown in Figure 3^b (65%) agrees closely with the fraction that is precipitable with SDS-K^+ (70%) in a paraliel reaction.

The purified enzyme was incubated with single strand DNA which had been 5' end labeled with ^{32}P. The enzyme was then denatured with 1% SDS thereby

Table II. Avian topoisomerase binds to 3' ends.

Reaction:	^{3}H % of Input SDS-K+ PPT:	^{32}P % of Input SDS-K+ PPT:
Avian topo	22.1	14.4
Procaryotic topo	12.5	1.4

The DNA substrates (^{3}H - DNA labeled by nick translation, 5 x 10^{6} dpm/ug and ^{32}P mononucleosome DNA, 1.5 x 10^{6} dpm/ug, labeled at the 5' end) were mixed and denatured with alkali (0.12 N NaOH, 15 min at 37°) followed by the addition of a pretitrated amount of 1 N HCl and 10 volumes of TEMP buffer to yield a final pH of 7.5. Reactions were assembled which contained denatured DNA (50 ng of ^{3}H and ^{32}P -DNA/ml) and either the avian topoisomerase I (170 ng/ml) or the procaryotic topoisomerase I (10 units/ml) in TEM buffer (final volume 0.1 ml). The reactions were incubated at 30° for 60 min and the SDS-K+ precipitable DNA measured as described in "Experimental Procedures." Each reaction was done in duplicate. Backgrounds were determined from reactions which were digested with proteinase K prior to the addition of KCl. Backgrounds were 1% (^{3}H) and 0.8% (^{32}P) of the input counts.

cross linking it to the helix. These complexes were separated from free DNA by the SDS-K^{+} precipitation method and radioactivity in the precipitate determined. As an internal control, nick translated ^{3}H -DNA was included in the reaction; in addition, the assay was also carried out with the purified procaryotic topoisomerase. The data in Table 2 show that both ^{3}H and ^{32}P-DNA were precipitated by the SDS-K^{+} method in the case of the avian topoisomerase, indicating that the enzyme binds to the 3' end. In reactions containing the procaryotic topoisomerase, only ^{3}H-DNA was detected in the SDS-K^{+} precipitate in agreement with previous reports indicating a 5' end association (12). When 3' end labeled DNA was used as substrate (prepared using DNA polymerase I Klenow fragment to fill recessed 3' ends of restriction fragments), the SDS-K^{+} assay data were also consistent with a 3' end association of the avian topoisomerase (data not shown).

Strand Transfer Activity. The rat liver and HeLa enzymes have been shown to promote the covalent transfer of heterologous DNA strands (2, 17). Incubation of the enzyme with single stranded DNA causes a decrease in the DNA chain length (17) and the formation of a DNA/protein complex in which the enzyme retains its ability to rejoin to an appropriate 'acceptor' DNA. We have tested for strand transfer activity of the avian topoisomerase using the electrophoretic assay described by Halligan et al. (17). In this analysis, plasmid DNA is nick translated, denatured and incubated with topoisomerase followed by incubation with an unlabeled 'acceptor' DNA. If the acceptor DNA

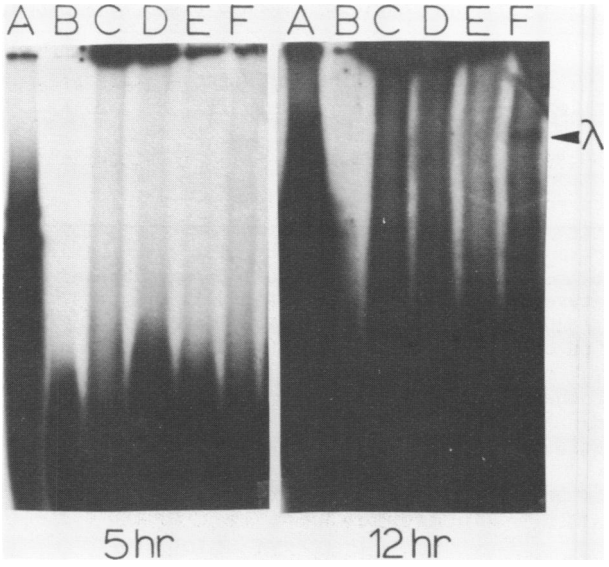

Figure 6. Strand Transfer Activity of Topoisomerase. Nick translated pBR-322 DNA (^{32}P labeled, 3 x 10^7 dpm/ug) was denatured with alkali, neutralized and placed on ice. Reactions containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 4 mM $MgCl_2$, 50 ug BSA/ml, 0.5 ug denatured ^{32}P-DNA/ml, 1.8 ug purified topoisomerase/ml (final volume 0.1 ml) were incubated at 30° for 30 min. (Aliquots were removed and SDS-K+ precipitable radioactivity determined as described in "Experimental Procedures".) Phage λ DNA, either treated or untreated with E. coli alkaline phosphatase, was then added to a final concentration of 8 ug/ml and incubations continued for 30 min at 30°. The reactions were digested with 50 ug proteinase K/ml after addition of SDS to 0.5%. Following ethanol precipitation, the DNA was resuspended in 10 mM NaOH, 1 mM EDTA, 10% glycerol, 0.1% bromophenol blue and loaded onto an agarose gel (0.8% in Tris-acetate buffer). After electrophoresis, the gel was dried and exposed to X-ray film for either 5 hr or 12 hr. Lane A: pBR-322 DNA prior to denaturation; Lane B: pBR-322 DNA after denaturation; Lane C: topoisomerase without added λ DNA; Lane D: alkaline phosphatase treated λ DNA, without topoisomerase; Lane E: λ DNA prior to treatment with alkaline phosphatase incubated with topoisomerase; Lane F: alkaline phosphatase treated λ DNA incubated with topoisomerase. In reactions which contained topoisomerase, approximately 20% of the input ^{32}P-DNA was precipitable by the SDS-K+ stoichiometric assay. The position of λ DNA as determined by ethidium bromide staining of the gel, is marked by an arrow.

can be distinguished from the single strand 'donor' DNA on the basis of size, it is possible to determine if the donor DNA has been covalently transferred using agarose gel electrophoresis. A strand transfer analysis is shown in Figure 6 where intact duplex λ DNA is used as the acceptor and nick translated pBR-322 DNA the single strand donor. Lanes A and B show the position of double and single strand pBR-322 donor DNA alone. Controls

included reactions without λ acceptor DNA (Lane C) or without enzyme (Lane D). The ^{32}P donor DNA was transferred only to λ acceptors which lacked 5' terminal phosphate (compare Lanes E and F) in agreement with previous reports (2, 17).

Topoisomerase Renders 25 bp of DNA Inaccessible to Micrococcal Nuclease. One aspect of topoisomerase/DNA interaction that is likely to be important in the distribution and function of the enzyme in chromatin is its binding site size on DNA. This characteristic reflects the number of nucleotides in DNA rendered inaccessible to further binding by the binding of a single enzyme molecule (31). To determine the binding site size the topoisomerase was incubated with ^{32}P -λ DNA (molar ratio of enzyme/DNA of 200) in low salt binding buffer and the native enzyme/DNA complex digested with micrococcal nuclease. As shown in Figure 7, DNA fragments accumulated, centered around 25 bp with a range of about 10 to 40 bp at 50% of peak intensity. With more extensive digestion, the size of the protected DNA fragments decreased slightly, as did the intensity. In a parallel reaction without topoisomerase, virtually all of the DNA was degraded within 30 seconds of digestion and labeled DNA fragments were not detected by autoradiography (not shown).

An additional fragment of nuclease-resistant DNA was detected in the experiment shown in Figure 7 (marked with an arrow). This DNA fragment of approximately 600 bp in length was present only in reactions which contained the topoisomerase. The significance of the 600 bp DNA is not clear at present and further studies are currently underway.

Discussion

The experiments show that an ATP, Mg^{++} independent topoisomerase is present in an avian cell line (1) and in erythrocytes, although the latter have less topoisomerase activity on a per cell basis. The reduced activity in erythrocytes is unlikely to be due to differences in growth rate since in mouse 3T3 cells, the specific activity of the enzyme is constant in G-1 arrested and actively growing cells suggesting that topoisomerase is not a cell cycle programmed function (11).

As a first step toward probing interaction between topoisomerase and chromatin, the enzyme has been purified from avian erythrocytes and its interaction with DNA investigated. The purification is rapid and the availability of large numbers ($>10^{12}$) of nuclei from chicken blood makes the purification convenient and economical. A peptide of molecular weight

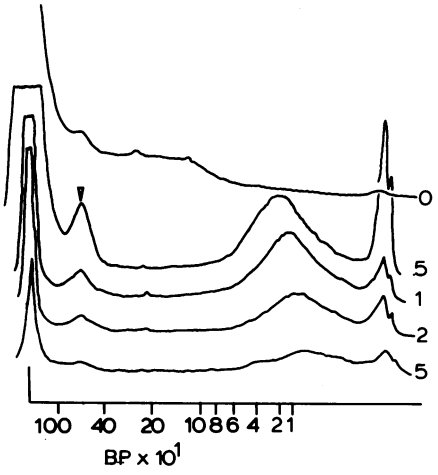

Figure 7. Topoisomerase Protects 25 bp of DNA from Micrococcal Nuclease Digestion. The reaction (150 ul) was assembled in LSB containing 1.66 ug ^{32}P -λ DNA/ml, 1.2 ug enzyme (fraction IV)/ml, and 50 ug BSA/ml. The enzyme had not been subjected to prior freeze/thaw cycles. After incubation at 30° for 30 minutes, the reaction was placed on ice and $CaCl_2$ added to 10 mM. The reaction was equilibrated to 37° for 1 minute and 0.25 units of micrococcal nuclease added. Digestions were terminated at 0, 0.5, 1, 2, and 5 minute intervals with 12.5 mM EDTA, 1% SDS followed by digestion with proteinase K. Aliquots of each time point were removed for determination of acid precipitable radioactivity and the remaining samples loaded on a 12% polyacrylamide slab gel prepared in Tris acetate buffer. Markers, consisting of Hae III or Hha I digested ØX-174 RFI DNA were analyzed in adjacent wells. The gel was stained with ethidium bromide, photographed, and dried prior to exposure to Kodak XAR-5 film for 26 hours. The negative was scanned with an LKB soft laser densitometer. The digestion times are indicated on the right of each tracing, and the molecular weight profile (in bp) on the abscissa. The total number of counts loaded/lane were: undigested, 2.8 x 10^4 ; 0.5 minutes, 6 x 10^3; 1 minute, 2.7 x 10^3; 2 minutes, 2 x 10^3; 5 minutes, 2.4 x 10^3. In a parallel reaction without topoisomerase, 95% of the ^{32}P -DNA was degraded in 0.5 minutes. No isotope was detected by autoradiography in the parallel reaction without topoisomerase after nuclease digestion (not shown).

105,000 was shown to copurify with the topoisomerase activity in close agreement with the recently reported molecular weights for the HeLa enzyme (22) and the wheat germ enzyme (13). Several lines of evidence support the idea that multiple forms of the topo exist which are catalytically active but differ in size. A reasonable interpretation of these data is that the various forms arise by cleavage of the M_r = 105,000 peptide. First, the lower molecular weight forms appear largely during the final stages of purification. Although the relative amounts of the different forms of

topoisomerase varied from one purification to the next, the M_r = 105,000 form was always present in the most pure fraction. Second, freezing and thawing (or extended dialysis) of the purified topo results in the loss of the higher molecular weight peptide and an accumulation of smaller peptides. Third, the various peptides in the final fraction displayed very similar chromatographic properties suggesting a common structural identity. Finally, we attempted to identify the multiple forms of topo more directly by cross linking the enzyme to ^{32}P-DNA, digesting unprotected DNA with nuclease and identifying proteins with attached ^{32}P-DNA tags by SDS-polyacrylamide gel electrophoresis (32). Four peptides were identified as being covalently coupled to DNA. Two of these (105 and 83,000) corresponded to the Coomassie stained peptides present in fraction IV; however, two additional ^{32}P labeled peptides (54 and 30,000) were not detected by Coomassie staining. Thus, the radioactivity associated with each peptide was not proportional to the amount of that peptide as detected by Coomassie staining. Such differences are not unexpected since the various peptides may protect the ^{32}P -DNA to different extents or reflect variable accessibility to nucleolytic cleavage. The experiment does identify a number of catalytically active forms of the avian topoisomerase I and suggests that the largest form is the M_r = 105,000. These data are in accord with previous studies with the HeLa enzyme which indicated that a lower molecular weight form of topoisomerase exists which is catalytically active (22). At the present time it is not clear which form of the topoisomerase predominates in vivo, nor what the physiological relationship is between the various forms; however, we have immunoprecipitated a M_r = 105,000 from MSB-1 nuclei using antibody to the purified avian topoisomerase (unpublished observations).

The avian topoisomerase I shares common biochemical features with other eucaryotic type I topoisomerases that have been investigated. The avian enzyme binds to single stranded DNA to yield a protein linked to the 3' end of the DNA (7, 17, 28). Under these conditions, the enzyme can covalently transfer the single strand DNA to a heterologous acceptor DNA containing 5' hydroxyl termini. For reasons that are not clear the transfer of single strand DNA is an inefficient process. The process may require ancillary proteins in chromatin, missing cofactors, or acceptor (or donor) DNA in a nucleosome configuration in order to function catalytically. Experiments are currently in progress to determine if nucleosomes will act as efficient acceptors in this reaction.

It is reasonable to assume that the distribution of topoisomerase in

chromatin is subject to constraints imposed by firmly bound structural proteins contained in the nucleosome. A second consideration which may be central to the distribution of the enzyme in chromatin is the binding site size of the enzyme. The binding site size, characterized by N, is the number of nucleotides rendered inaccessible to further binding after a single molecule of topoisomerase is bound (for review see 31). We have used topoisomerase mediated nuclease protection experiments to approximate the value of N, much in the same way that nucleases have been successfully used to define the binding and wrapping of the helix about the inner histone core of the nucleosome (for review see 14). The topoisomerase protects roughly 25 bp from micrococcal nuclease cleavage, suggesting N = 25; although the range of fragment lengths was rather broad (average of ∿10 to 40 bp). Fragment length heterogeneity may be due to sliding or dissociation and rebinding of the native topoisomerase during nuclease treatment. Other lines of supportive data suggest that our estimate of N is a reasonable one. Recently, the Stokes radius for the HeLa topoisomerase I was estimated to be 50 Å (22). Assuming a similar radius for the avian enzyme, the binding of a roughly spherical protein of this size to DNA could sterically preclude nuclease access to the helix over a linear span of ∿30 bp, in close agreement with our estimate of N.

Although we have shown no data concerning the wrapping of protein about the helix, or vice versa, other experiments not shown here, using DNase I to digest the native topoisomerase/DNA complex yielded essentially similar results to the micrococcal nuclease experiments. No evidence was found for a 10 bp periodicity as demonstrated with DNA gyrase (23). These data suggest that the DNA is primarily buried within the enzyme molecule and argue against wrapping of the helix on the surface of the topoisomerase.

It is of interest that the binding site size of topoisomerase I (25 bp) is compatible with the idea that the binding of topoisomerase in chromatin may be confined to linker regions between nucleosome core particles. Recent experiments in this laboratory have shown that the endogenous topoisomerase co-sediments with identifiable nucleosome subunits in low ionic strength sucrose gradients; in addition, isolated mononucleosomes bind significantly less exogenously added enzyme than protein free DNA of the same size (Muller and Trask, in preparation). These observations support the idea that the inner histone core structure sterically blocks topoisomerase binding and that the enzyme interacts with DNA external to the core particle.

ABBREVIATIONS USED

EDTA, ethylenediaminetetraacetic acid; bp, base pairs; SDS, sodium dodecyl sulfate; RBC, red blood cell; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; form I DNA, covalently closed supercoiled DNA; form II DNA, open circular DNA; form III DNA, linear DNA.

ACKNOWLEDGEMENTS

We thank Dr. J.N. Reeve and Dr. D. Ives for a critical reading of the manuscript and for helpful discussions. The technical assistance of J. Shields was greatly appreciated. This work was supported in part by grant No. R01 GM316401-01 from the National Institute of Health.

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