

- Janik, B., Kotik, M. P., Kreiser, T. H., Reverman, L. F., Sommer, R. G., & Wilson, D. P. (1972) *Biochem. Biophys. Res. Commun.* 46, 1153-1160.
- Jeffries, A. C., & Symons, R. H. (1989) *Nucleic Acids Res.* 17, 1371-1377.
- Kakiuchi, N., Marek, C., Rousseau, N., Leng, M., De Clercq, E., & Guschlbauer, W. (1982) *J. Biol. Chem.* 257, 1924-1928.
- Koizumi, M., Iwai, S., & Ohtsuka, E. (1988) *FEBS Lett.* 239, 285-288.
- Koizumi, M., Hayase, Y., Iwai, S., Kamiya, H., Inoue, H., & Ohtsuka, E. (1989) *Nucleic Acids Res.* 17, 7059-7071.
- Kuchino, Y., & Nishimura, S. (1989) *Methods Enzymol.* 180, 154-163.
- Mei, H.-Y., Kaaret, T. W., & Bruice, T. C. (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86, 9727-9731.
- Middleton, W. J. (1975) *J. Org. Chem.* 40, 574-578.
- Morishita, K., Hakoshima, T., Fujiwara, T., Tomita, K., Kaneyasu, T., Uesugi, S., & Ikehara, M. (1984) *Acta Crystallogr., Sect. C* 40, 434-436.
- Perreault, J.-P., Wu, T., Cousineau, B., Ogilvie, K. K., & Cedergren, R. (1990) *Nature* 344, 565-567.
- Perreault, J.-P., Labuda, D., Usman, N., Yang, J.-H., & Cedergren, R. (1991) *Biochemistry* 30, 4020-4025.
- Pieken, W. A., Olsen, D. B., Benseler, F., Aurup, H., & Eckstein, F. (1991) *Science* 253, 314-317.
- Ranganathan, R. (1977) *Tetrahedron Lett.* 15, 1291-1294.
- Rossi, J. J. & Sarver, N. (1990) *Trends Biotechnol.* 8, 179-183.
- Ruffner, D. E., & Uhlenbeck, O. C. (1990) *Nucleic Acids Res.* 18, 6025.
- Ruffner, D. E., Stromo, G. D., & Uhlenbeck, O. C. (1990) *Biochemistry* 29, 10695-10702.
- Saenger, W., Ed. (1984) in *Principles of Nucleic Acid Structure*, Springer Verlag, New York, Berlin, Heidelberg, and Tokyo.
- Sarver, N., Cantin, E. M., Chang, P. S., Zaia, J. A., Ladne, R. A., Stephens, D. A., & Rossi, J. J. (1990) *Science* 247, 1222-1224.
- Scaringe, S. A., Francklyn, C., & Usman, N. (1990) *Nucleic Acids Res.* 18, 5433-5441.
- Sheldon, C. C., & Symons, R. H. (1989) *Nucleic Acids Res.* 17, 5679-5685.
- Sheldon, C. C., Jeffries, A. C., Davies, C., & Symons, R. H. (1990) in *Nucleic Acids and Molecular Biology* (Eckstein, F., & Lilley, D. M. J., Eds.) pp 227-242, Springer-Verlag, Berlin, Heidelberg.
- Sinha, N. D., Biernat, J., & Köster, H. (1984) *Nucleic Acids Res.* 12, 4539-4557.
- Slim, G., & Gait, M. J. (1991) *Nucleic Acids Res.* 19, 1183-1188.
- Tinoco, I., Puglisi, J. D., & Wyatt, J. R. (1990) in *Nucleic Acids and Molecular Biology* (Eckstein, F., & Lilley, D. M. J., Eds.) pp 204-226, Springer-Verlag, Berlin, Heidelberg.
- Tsuchiya, T. (1990) in *Advances in Carbohydrate Chemistry and Biochemistry* (Tipson, R. S., & Horton, D., Eds.) pp 91-277, Academic Press, San Diego.
- Uchida, T., & Egami, F. (1971) in *Hydrolysis* (Boyer, P. D., Ed.) pp 205-250, Academic Press, New York and London.
- Uesugi, S., Miki, H., Ikehara, M., Iwahashi, H., & Kyogoku, Y. (1979) *Tetrahedron Lett.* 42, 4073-4076.
- Uhlenbeck, O. C. (1987) *Nature* 328, 596-600.
- Williams, D. M., Benseler, F., & Eckstein, F. (1991) *Biochemistry* 30, 4001-4009.

## DNA Topoisomerase I Cleavage Sites in DNA and in Nucleoprotein Complexes<sup>†</sup>

Arndt Richter\* and Jürgen Ruff

Division of Biology, University of Konstanz, D-7550 Konstanz, FRG

Received April 22, 1991; Revised Manuscript Received July 15, 1991

**ABSTRACT:** The intracellular substrate for eukaryotic DNA topoisomerases is chromatin rather than protein-free DNA. Yet, little is known about the action of topoisomerases on chromatin-associated DNA. We have analyzed to what extent the organization of DNA in chromatin influences the accessibility of DNA molecules for topoisomerase I cleavage in vitro. Using potassium dodecyl sulfate precipitation (Trask et al., 1984), we found that DNA in chromatin is cleaved by the enzyme with somewhat reduced efficiency compared to protein-free DNA. Furthermore, using native SV40 chromatin and mononucleosomes assembled in vitro, we show that DNA bound to histone octamer complexes is cleaved by topoisomerase I and that the cleavage sites as well as their overall distribution are identical in histone-bound and in protein-free DNA molecules.

**T**ranscription and replication of cellular DNA require the separation of complementary strands of the DNA double helix. This unwinding in topologically fixed molecules causes a coiling of the helix axis behind and/or in front of the advancing transcription complex or replication fork. These topological constraints may inhibit both processes and are relaxed by

topoisomerases. It is widely accepted that topoisomerase I is one of the major cellular enzymes performing this function in transcription and replication [summarized in Wang (1987), Liu (1989), Richter and Knippers (1989), Hsieh (1990), and Austin and Fisher (1990)].

The catalytic activity of eukaryotic topoisomerase I has been extensively characterized (Vosberg, 1985; Wang, 1985). The enzyme initiates the reaction cycle by binding to the substrate DNA followed by the introduction of a nick into one strand

<sup>†</sup> This work was supported by the Deutsche Forschungsgemeinschaft through Grant SFB 156.

of the double-stranded DNA molecule. As a reaction intermediate, a covalent protein–DNA complex is formed via a phosphodiester bond between the “active site tyrosine” (Lynn et al., 1989; Eng et al., 1989) of the enzyme and the 3' end of the broken DNA strand. Topological tension is released at the site of strand discontinuity, and finally the nick is resealed on expense of the energy trapped in the covalent protein–DNA linkage. Topoisomerase I changes the linking number of DNA duplexes in steps of 1, thus removing positive and negative superhelical turns in topologically fixed DNA molecules.

Three structural parameters can affect the enzymatic activity of topoisomerase I, namely, the nucleotide sequence of the DNA, the superhelical density of the substrate molecule, and the structure and protein architecture of the chromosomally organized DNA. We now consider these points in more detail.

(i) If a mixture of topoisomerase I and DNA is denatured by alkali or sodium dodecyl sulfate, the strand scissions introduced by the enzyme into the DNA are irreversibly trapped, and the covalent complex between enzyme and DNA persists. The denaturant-stabilized cleavage sites are assumed to reflect those sites where topoisomerase I binds and carries out its catalytic action. The sequence requirements for the cleavage reaction catalyzed by topoisomerase I have been examined, and two types of cleavage sites have been established. A high-affinity cleavage site was found clustered in the promoter region of *Tetrahymena* rRNA genes (Andersen et al., 1985; Bonven et al., 1985; Christiansen et al., 1987). Other cleavage sites present on a variety of viral and cellular DNA fragments have been identified, and several partly divergent consensus sequences for the topoisomerase I cleavage reaction have been suggested (Edwards et al., 1982; Been et al., 1984; Perez-Stabel et al., 1988; Porter & Champoux, 1989).

(ii) Relaxed covalently closed circular DNA molecules as well as linear DNA are substrates for the topoisomerase I cleavage reaction. The efficiency of the cleavage reaction on covalently closed supercoiled circular DNA is, however, several orders of magnitude higher when compared to the cleavage reaction on the same relaxed or linearized DNA molecule (Camilloni et al., 1989; Caserta et al., 1990). This suggests that the superhelical tension in DNA molecules is another important determinant of topoisomerase I activity in vitro and most likely in vivo as well (Giaever et al., 1988).

(iii) Little is known about the influence of chromatin structure on the sequence recognition by eukaryotic topoisomerase I. In this paper, we have addressed the question of whether the organization of DNA as nucleoprotein complexes affects the DNA cleavage reaction catalyzed by topoisomerase I. We show that topoisomerase I has access to DNA in nucleoprotein complexes and cleaves the DNA packaged into nucleosomes and chromatin at the same specific sites as in protein-free DNA.

#### EXPERIMENTAL PROCEDURES

**Materials.** Chemicals were from Merck/Darmstadt, camptothecin (lactone form; CPT) was from Sigma Chemie/Deisenhofen, biochemicals and enzymes were from Boehringer/Mannheim, and radionucleotides were from Amersham/Braunschweig. Topoisomerase I was prepared from HeLa cell nuclei and calf thymus as described (Strausfeld & Richter, 1989). The 154 base pair (bp)<sup>1</sup> long *EcoRI*/*NotI*

DNA fragment is from the promoter region of the topoisomerase I gene (Kunze et al., 1990), and the 206 bp fragment is the small *EcoRI*/*PstI* fragment from SV40 DNA containing nucleotides 1783–1988. The fragments were labeled with Klenow polymerase in the presence of [<sup>32</sup>P]dATP at the *EcoRI* site and purified by gel filtration and ethanol precipitation.

**Preparation of SV40 Chromatin.** SV40 minichromosomes were extracted from nuclei of infected African Green monkey kidney cells (TC 7 cells) 48 h after infection as described (Sundin & Varshavsky, 1980). Extracted minichromosomes were further purified on 7.5–30% sucrose gradients (Richter et al., 1987) containing 450 mM sodium chloride. SV40 chromatin prepared in this way was free of histone H1, DNA topoisomerases I and II, and the majority of other non-histone chromatin proteins associated with SV40 chromatin in low-salt extracts.

**Preparation of Mononucleosomes.** Mononucleosomes were assembled on a 154 bp and on a 206 bp long DNA fragment by two different methods. Method 1: The histone transfer method described by Drew and Travers (1987) was adopted to assemble mononucleosomes with isolated SV40 minichromosomes as nucleosome donors. Briefly, the end-labeled DNA fragments were incubated with a 200-fold molar excess of SV40 chromatin in TE buffer (10 mM Tris, pH 7.5, and 1 mM NaEDTA) containing 1 mM dithiothreitol (DTT), 0.05 mg/mL bovine serum albumin, and 800 mM sodium chloride for 1 h at 37 °C. The salt concentration was then reduced stepwise to 700, 600, 500, 400, 300, and 150 mM sodium chloride by dilutions with 10 mM Tris, pH 7.5, and 1 mM NaEDTA in 15-min intervals. After dilution, the samples were adjusted to 400 mM sodium chloride and 0.1% Nonidet P40, and the mononucleosomes formed were separated from excess donor chromatin and free DNA by centrifugation through 5–25% linear sucrose gradients made in 10 mM Tris-HCl (pH 7.4), 1 mM NaEDTA, 1 mM DTT, and 0.05 mg/mL bovine serum albumin (BSA) for 16 h at 36 000 rpm and 0 °C in a Beckman SW55 rotor. Method 2: Nucleosomes were assembled by incubation of labeled DNA in *Xenopus* egg extracts according to the procedure described by Kleinschmidt et al. (1982) and purified as described above.

The stability of each preparation was monitored by recentrifugation after incubation for 15 min at 20 °C with topoisomerase I in 20 mM Tris-HCl, pH 7.5, 75 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT, and 0.05 mg/mL BSA (not shown) and gel electrophoresis in 4% polyacrylamide gels [acrylamide:bis(acrylamide) ratio 40:1; 0.5 × TBE buffer; Maniatis et al., 1982]. Digestion with micrococcal nuclease revealed that the mononucleosomal DNA was completely resistant under conditions where 80% of protein-free DNA was rendered acid-soluble (data not shown).

**Topoisomerase I Cleavage Reaction.** In a standard cleavage reaction, the indicated amounts of the labeled substrates were incubated with purified topoisomerase I in a total of 25 µL of cleavage buffer (20 mM Tris-HCl, pH 7.5, 75 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT, and 0.05 mg/mL BSA) for 15 min at 20 °C. Camptothecin in dimethyl sulfoxide (DMSO) or DMSO or H<sub>2</sub>O alone was included in the reaction as indicated in the figure legends. After incubation for 15 min at 20 °C, 200 µL of 0.5% sodium dodecyl sulfate in TE was added to stop the reaction.

**Potassium Dodecyl Sulfate Precipitation of Covalent Topoisomerase I–DNA Complexes.** Protein–DNA complexes formed upon addition of sodium dodecyl sulfate to the cleavage reaction were precipitated on ice by the addition of potassium chloride to a final concentration of 250 mM. The precipitate

<sup>1</sup> Abbreviations: bp, base pair(s); BSA, bovine serum albumin; DTT, dithiothreitol; NaEDTA, sodium ethylenediaminetetraacetate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

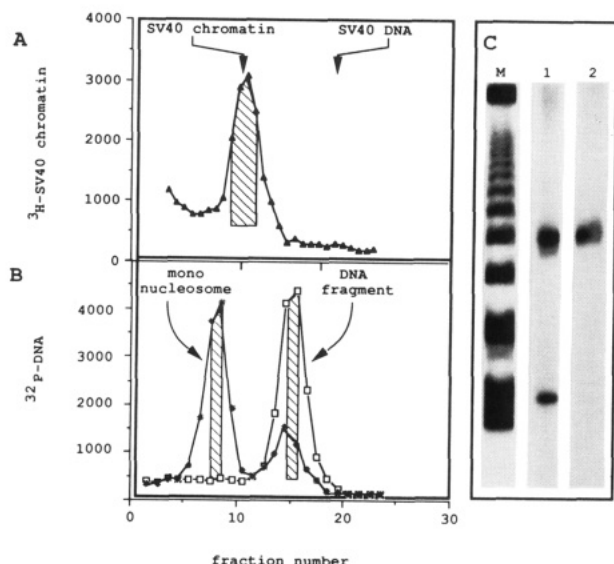


FIGURE 1: Nucleoprotein substrates. (A) SV40 chromatin. Sucrose gradient purification of SV40 chromatin labeled with [ $^3\text{H}$ ]thymidine in vivo. Closed symbols, SV40 DNA; striped box, SV40 chromatin pool. (B) Mononucleosomes. Sucrose gradient purification of the mononucleosomes assembled with the 154 bp DNA fragment end-labeled with [ $^{32}\text{P}$ ]dATP. Open symbols, free 154 bp DNA fragment; closed symbols, 154 bp DNA fragment after the histone transfer reaction; striped boxes, mononucleosome and DNA pools. (C) Nondenaturing polyacrylamide gel electrophoresis of the products of a histone transfer reaction. M, 123 bp ladder; lane 1, products of the histone transfer reaction (lower band, free 154 bp DNA fragment; upper band, mononucleosome); lane 2, purified mononucleosomes after sucrose gradient centrifugation.

formed was pelleted (2000g, 2 min, room temperature) and washed 3 times in 250  $\mu\text{L}$  of TE containing 250 mM potassium chloride. The final pellet was dissolved in 100  $\mu\text{L}$  of 0.1 M HCl, and the radioactivity was determined by liquid scintillation counting in 1 mL of aquasol (DuPont).

**Determination of Topoisomerase Cleavage Sites.** Topoisomerase I cleavage reactions were performed as described above. After treatment with proteinase K for 2 h at 50  $^{\circ}\text{C}$ , the DNA fragments were phenol-extracted, ethanol-precipitated, and analyzed on 4% polyacrylamide/urea gels with end-labeled SV40 *Hind*III fragments as length markers (large SV40 fragments) or on 4% sequencing gels (small fragments). In the latter case, the same labeled DNA fragment treated in a Maxam-Gilbert GA reaction was separated in parallel as a marker.

**Gel Electrophoresis and Autoradiography.** Gel electrophoresis on agarose and polyacrylamide gels was performed as described (Maniatis et al., 1982). The dried gels were exposed to Kodak X-ray films.

## RESULTS

**Purification of Native SV40 Chromatin and Mononucleosome Assembly.** We have analyzed the interaction of purified human and calf thymus topoisomerase I with free DNA and two forms of nucleoprotein complexes, namely, SV40 chromatin and in vitro assembled mononucleosomes. Figure 1 shows the purification of the nucleoprotein complexes which we used as substrates in the topoisomerase I reaction. Native SV40 chromatin was isolated from nuclei of infected TC7 cells (Sundin & Varshavsky, 1980) and was purified by sucrose gradient centrifugation (Figure 1A). The purified chromatin was then used either as nucleosome donor in histone transfer reactions or as a substrate for topoisomerase I. Mononucleosomes, the second type of nucleoprotein complexes, were assembled on a 206 bp and on a 154 bp long DNA

fragment, respectively, by two different methods (see Experimental Procedures). Figure 1B shows, as an example, the products of an assembly reaction performed according to the histone transfer method with the 154 bp DNA fragment and purified SV40 chromatin (shown in Figure 1A) as a nucleosome donor. As demonstrated by sucrose gradient centrifugation (Figure 1B) and nondenaturing polyacrylamide gel electrophoresis (Figure 1C), stable mononucleosomes are formed during the transfer reaction. Similar results were obtained when *Xenopus laevis* oocyte extracts were used for nucleosome assembly (not shown). Fractions containing SV40 chromatin or mononucleosomes were combined from the sucrose gradients (Figure 1A,B) and were used directly for the topoisomerase I reactions described below.

**Interaction of Topoisomerase I with DNA and DNA in Nucleoprotein Complexes.** We performed potassium dodecyl sulfate coprecipitation of enzyme-DNA complexes to determine the interaction between topoisomerase I and SV40 chromatin or purified linear SV40 DNA. Addition of potassium chloride to the sodium dodecyl sulfate treated assay mixture results in the formation of a precipitate which can be pelleted by low-speed centrifugation. Since dodecyl sulfate binds proteins, protein- and detergent-resistant protein-DNA complexes are readily separated from free DNA which remains in the supernatant. This method allows a quantitative measurement of the amount of DNA that is covalently coupled with protein (Trask et al., 1984; Rowe et al., 1986).

For the experiments, [ $^3\text{H}$ ]thymidine-labeled SV40 chromatin was prepared as described above. SV40 DNA was then purified from half of the chromatin fraction, and the DNA was linearized by restriction nuclease *Eco*RI. In the experiments reported below, camptothecin was used to stabilize the covalent reaction intermediate formed between topoisomerase I and DNA. Increasing amounts of topoisomerase I were incubated either with chromatin containing 100 ng of SV40 DNA or with the same amount of protein-free SV40 DNA at 37  $^{\circ}\text{C}$  for 20 min in the presence of 20  $\mu\text{M}$  camptothecin. The reactions were terminated by addition of sodium dodecyl sulfate, and the formation of covalent complexes was determined. As shown in Figure 2A, there is a linear increase of the amount of enzyme-DNA complexes formed during incubation with increasing amounts of topoisomerase I. About 30% more protein-free DNA than DNA in chromatin is precipitated by a given amount of enzyme. This result indicates a higher affinity of the enzyme for protein-free DNA or a better accessibility of the protein-free DNA molecules compared to DNA in chromatin. Most covalent enzyme-DNA complexes are formed rapidly within 2 min after addition of topoisomerase I followed by a continuous but very slow increase of complexes formed during further incubation (Figure 2B). When camptothecin was omitted and the reaction was performed in the presence of DMSO or with water, the amount of complexes formed was reduced by a factor of 10. However, as in the presence of camptothecin, about 25–30% more complexes were formed between enzyme and free DNA when compared with chromatin or mononucleosomes (not shown).

In agreement with the data reported previously (Camilloni et al., 1989; Caserta et al., 1990), the amount of enzyme-DNA complexes formed with supercoiled free SV40 DNA at a given enzyme concentration was 7-fold higher under our assay conditions when compared with the amount of complexes formed with linearized DNA molecules (not shown). No DNA was present in the precipitate when topoisomerase I was omitted, or when the samples were treated with proteinase K prior to precipitation.

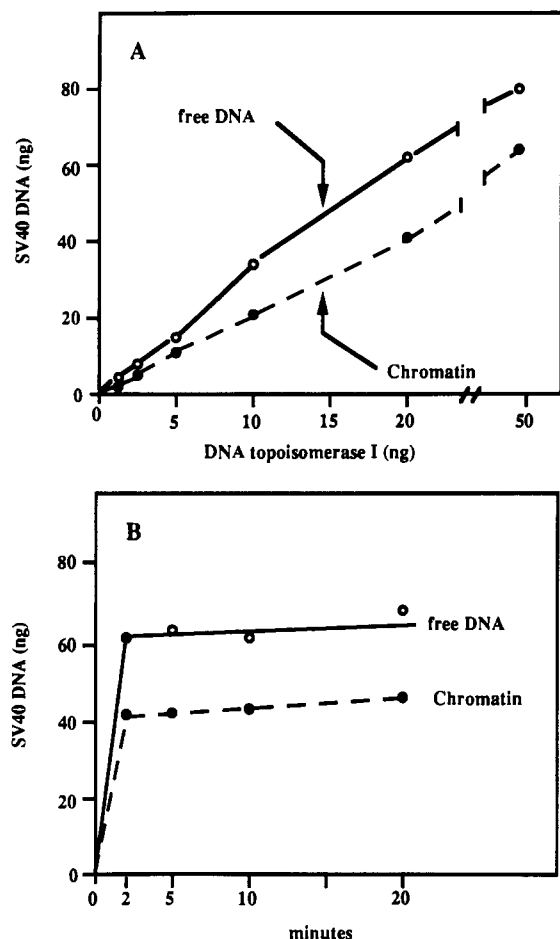


FIGURE 2: Potassium chloride precipitation of covalent topoisomerase I-SV40 DNA complexes. (A) Increasing amounts of topoisomerase I were incubated with 100 ng of linearized SV40 DNA or the same amount of SV40 DNA in chromatin. After incubation for 15 min at 37 °C, the amount of potassium dodecyl sulfate precipitable radioactivity was determined. (B) Five nanograms of topoisomerase I was incubated with 100 ng of linearized SV40 DNA of the same amount of SV40 DNA in chromatin for 0, 2, 5, 10, and 25 min at 37 °C, and the amount of potassium dodecyl sulfate precipitable radioactivity was determined. The reactions shown in (A) and (B) were performed in the presence of 20  $\mu$ M camptothecin in DMSO (0.1% final). Open symbols, DNA; closed symbols, chromatin.

We have repeated this experiment with mononucleosomes assembled on a 154 bp or a 206 bp long DNA fragment by the histone transfer method (Figure 1B). As in the case of SV40 DNA and chromatin, 25% less mononucleosomal DNA was precipitated compared to protein-free DNA (Figure 3). The kinetics of the reactions were essentially identical with the kinetics determined with SV40 DNA and chromatin with a rapid complex formation within the first 2 min and a further slow increase in their amount during prolonged incubation (not shown).

**Determination of Topoisomerase I Cleavage Sites in SV40 DNA and SV40 Chromatin.** We have determined topoisomerase I cleavage sites in DNA and in native chromatin within the 1014 bp long *EcoRI/EcoRV* fragment of SV40 DNA. The *EcoRV/EcoRI* fragment of SV40 was cloned into the *EcoRI/SmaI* sites of pUC18, resulting in plasmid pUCSV. This plasmid (100 ng) was linearized with *EcoRI*, combined with the same amount of gradient-purified SV40 chromatin, and incubated with 50 ng of topoisomerase I for 15 min at 37 °C. The reaction was terminated with sodium dodecyl sulfate and proteinase K digestion. The purified DNA containing circular SV40 DNA and linearized pUCSV DNA was then digested with *EcoRI* and *SphI*, resulting in a 1045 bp long

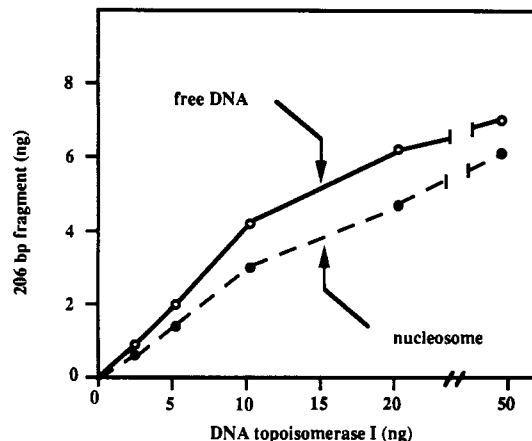


FIGURE 3: Potassium chloride precipitation of covalent topoisomerase I-DNA complexes. Increasing amounts of topoisomerase I were incubated with 10 ng of a 206 bp DNA molecule labeled with [ $^{32}$ P]dATP or the same amount of DNA assembled into mononucleosomes according to the histone transfer method. After incubation for 15 min at 37 °C, the amount of potassium dodecyl sulfate precipitable radioactivity was determined. The reactions were performed in the presence of 20  $\mu$ M camptothecin in DMSO (0.1% final). Open symbols, DNA; closed symbols, nucleosome.

fragment derived from pUCSV and a 1582 bp long fragment derived from SV40 chromatin (Figure 4A). The resulting fragments were end-labeled at the *EcoRI* site with [ $^{32}$ P]dATP, and the two fragments containing the *EcoRI/EcoRV* fragment of SV40 DNA were separated and isolated from agarose gels. The two fragments were then heat-denatured and analyzed on a 4% polyacrylamide/urea gel. The subfragments resulting from cleavages introduced by topoisomerase I are shown in Figure 4B, and a densitometric evaluation is shown in Figure 4C. Strong topoisomerase I induced cleavage sites were found distributed over the entire length of the SV40-derived DNA fragments. On the level of resolution of the gel, all cleavages introduced in the free 1045 bp DNA fragment are also present in the 1582 bp DNA fragment derived from chromatin. An analysis of the terminal nucleotides present in both fragments on 4% sequencing gels confirmed these results and revealed the cleavage site identity on the sequence level (not shown). Essentially identical results were obtained when the topoisomerase I cleavage reaction was performed with SV40 DNA in chromatin or plasmid pUCSV DNA separately.

**Determination of Topoisomerase I Cleavage Sites in Mononucleosomes.** The identity of cleavage sites on the sequence level was also found when protein-free DNA was compared with DNA assembled into mononucleosomes. Figure 5 (lanes 1-6) shows the characterization of cleavage sites on the 154 bp long protein-free DNA fragment. Strong cleavage sites were present at the lowest enzyme concentration used (Figure 5, lane 2; see, for example, cleavage site c) whereas weaker cleavage sites were only detected at higher enzyme concentrations (lanes 2-4; see, for example, the cleavage sites e, g, h, and m). Two types of cleavage sites can be further distinguished, namely, sites cleaved in the absence of the drug (Figure 5, compare lane 4 with lanes 5 and 6; cleavage sites c, h, and k) and other sites where cleavage is enhanced or even strongly enhanced by the drug (Figure 5, lanes 2-4; cleavage sites a, b, d, e, f, g, i, l, and m). These two types of sites have been found before in a variety of fragments from different sources (Kjeldsen et al., 1988; Porter & Champoux, 1989).

In Figure 5 (lanes 7-10), the cleavages introduced into the 154 bp protein-free and the nucleosome-associated DNA fragment are compared. The cleavage reaction was performed in the presence of camptothecin. All topoisomerase I cleavage

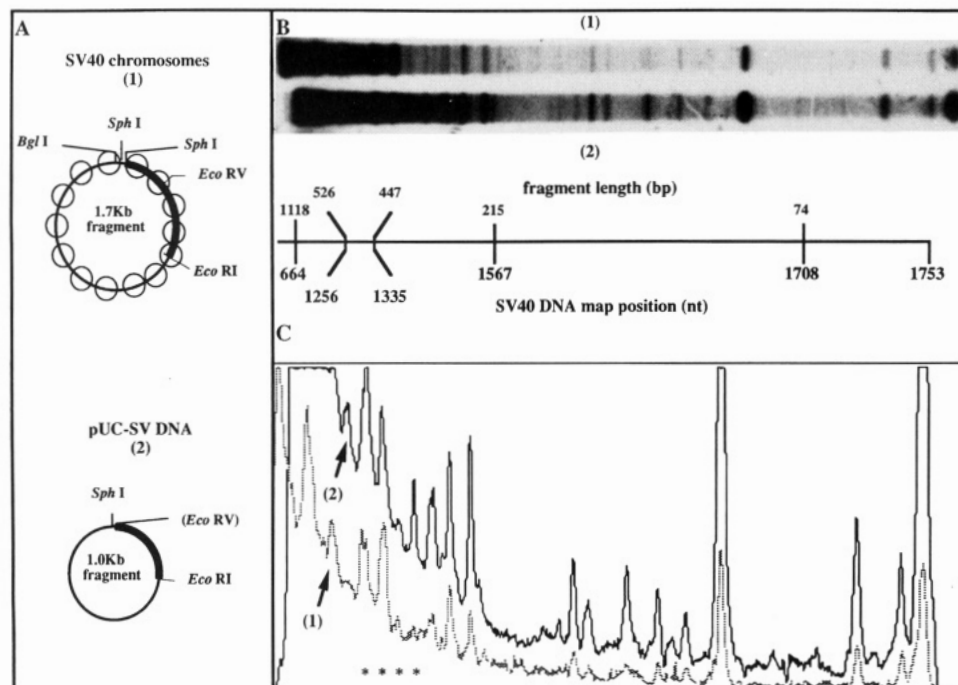


FIGURE 4: Analysis of topoisomerase I cleavage sites in a SV40 DNA fragment and the same fragment from SV40 chromatin. (A) DNA substrates used for topoisomerase I cleavage. (1) SV40 chromosomes. (2) Plasmid pUCSV containing the *EcoRV*/*EcoRI* fragment (nucleotide 769 to nucleotide 1782) of SV40 DNA in pUC18. Restriction enzyme cleavage sites used are indicated. Boldface segments, fragments analyzed in (B) and (C). (B) Fifty nanograms each of linearized pUCSV DNA and 50 ng of SV40 DNA in chromatin (see panel A) were incubated with 50 ng of topoisomerase I for 15 min at 37 °C, and the DNAs were isolated. The purified DNAs were digested with *EcoRI* and *SphI* and end-labeled at the *EcoRI* cleavage site with [<sup>32</sup>P]dATP. The 1045 bp fragment derived from pUCSV DNA and the 1582 bp fragment derived from SV40 chromatin were purified by agarose gel electrophoresis and separated on 4% polyacrylamide/urea gels under denaturing conditions. The scale below the autoradiography indicates the length of the DNA fragments and the cleavage positions on the SV40 map. Panel C shows the densitometric evaluation of the reaction products shown in (B). The asterisks mark topoisomerase I cleavages present within a region of strong nucleosome binding as determined by Ambrose et al. (1990; see Discussion).

sites determined in protein-free DNA were cleaved in mononucleosomal DNA as well. In agreement with the data shown in Figure 3, cleavage was suppressed when nucleosomal DNA (lane 8; 25% uncleaved fragment) was compared to protein-free DNA (lane 10; 16% uncleaved fragment). A densitometric evaluation showed that cleavage at sites b, c, and d (as examples) was by a factor of 1.5 more frequent in mononucleosomes than in DNA. Conversely, cleavages in sites f, i, and m were found by a factor of 1.6 more frequent in DNA compared to mononucleosomes. These differences most likely do not reflect an altered affinity of topoisomerase I for certain cleavage sites in mononucleosomes but rather result from the better accessibility of cleavage sites in free DNA. This better accessibility of sites in free DNA resulted in a bias toward smaller fragments when the DNA was cleaved more than once. We have repeated this experiment with the 206 bp fragment derived from SV40 DNA as well as with DNA from other sources and obtained similar results (not shown).

The experiments shown above were performed with mononucleosomes assembled according to the histone transfer method with SV40 chromatin as a nucleosome donor. To confirm this result and to determine whether the source of histones and the method of nucleosome assembly may affect the accessibility of the underlying DNA for topoisomerase I cleavage, we used a nonmammalian extract and a different method to assemble the mononucleosomes *in vitro*. As shown in Figure 6, direct comparison of the products of the cleavage reaction with mononucleosomes assembled by the histone transfer method (Figure 6, lanes 1–6) and with mononucleosomes assembled in extracts derived from oocytes of *X. laevis* (Figure 6, lanes 7–12) reveals that in both cases topoisomerase I interacts with the DNA at the same sites and with similar efficiencies as compared with protein-free DNA. The

cleavage site specificity of the enzyme remains unaffected as camptothecin-independent and camptothecin-enhanced cleavages are found at identical sites in nucleosomes and in free DNA (Figure 6, lanes 13–18). Furthermore, at least under our experimental conditions no additional sites are induced by the organization of DNA into a nucleosome. A summary of the topoisomerase I cleavage sites is given in Figure 7. These sites were identified on the 154 bp long DNA fragment regardless of whether the fragment was used as a protein-free substrate or in association with a histone octamer. Furthermore, identical cleavages were obtained with mammalian topoisomerase I from human and calf thymus in DNA and DNA in nucleosomes formed from mammalian and nonmammalian histones.

## DISCUSSION

In eukaryotic cells, the DNA is wrapped around nucleosomes with the DNA helix exposed on the outer surface of the nucleosome [for a review, see Morse and Simpson (1988)]. The intimate association of the histone proteins with the DNA and the close proximity of the two turns of the helix to each other could provide steric and ionic barriers sufficient to render the DNA inaccessible to interaction with other proteins. The question of protein–chromatin interactions is therefore an important one since chromatin is the substrate upon which all nuclear enzymes in eukaryotes must operate (Ness et al., 1988). In this paper, we have investigated whether the organization of DNA in nucleoprotein complexes influences the interaction of topoisomerase I with the DNA substrate.

*In vivo* topoisomerase I cleavage sites have been mapped in chromatin of *Xenopus laevis* (Culotta & Sollner-Webb, 1988) and in *Dictyostelium* ribosomal RNA genes (Ness et al., 1988). In *X. laevis* chromatin, topoisomerase I cleavage





gions. In *Dictyostelium*, extensive topoisomerase I cleavage activity is found across the whole coding region of 17S, 5.8S, 26S, and 5S rRNA as well as in the downstream spacer regions. The resolution of the gels used gave no evidence for a regular spacing of cleavages within the coding region, but some of the strongest cleavages found in the transcribed spacer regions are clearly located in irregular distances from each other. Furthermore, it has been shown that during transcription of RNA polymerase II genes topoisomerase I specifically interacts with the coding region, and cleavage sites have been roughly localized (Gilmore & Elgin, 1987; Rowe et al., 1987; Stewart et al., 1990). However, no apparent regular spacing of these cleavage sites has been reported.

Using an assay which quantitatively measures the formation of covalent topoisomerase I-DNA reaction intermediates, we have demonstrated that the accessibility of DNA in *in vivo* formed SV40 chromatin as well as in *in vitro* assembled nucleosomes is suppressed when compared with protein-free DNA. This suppression, however, does not result in the complete exclusion of the enzyme from sites in DNA which are covered by nucleosomes. It could be argued that the reduced formation of covalent complexes between topoisomerase I and SV40 chromatin (see Figure 2) reflects an exclusion of the enzyme from DNA sites bound by nucleosomes whereas in spacer regions between individual nucleosomes the DNA is accessible for the enzyme. The fact, however, that we obtain no stronger suppression in experiments with mononucleosomes assembled on a 206 bp (see Figure 3) or a 154 bp long DNA fragment *in vitro* makes this possibility unlikely as, at least in case of the latter substrate, the cleavable complex is formed with DNA which is completely bound to the nucleosome.

We have compared the cleavages by topoisomerase I into a 1014 bp long DNA sequence present in isolated SV40 minichromosomes with cleavages introduced into the same sequence present as protein-free DNA and found that topoisomerase I cleaves at the same specific sites and with comparable efficiencies in DNA covered with nucleosomes and in protein-free DNA.

The location of nucleosomes in SV40 chromatin has been analyzed by Ambrose et al. (1990). The authors established that the SV40 genome contains regions which were stably and preferentially occupied by nucleosomes and other regions which were not. Among the strong SV40 nucleosome location sites, one site, designated VI-2 [see Ambrose et al. (1990)], is contained in the SV40 region analyzed in this study and indicated by asterisks in Figure 4C. In this region, cleavages were present at the same sites in protein-free DNA and in chromatin. Though we cannot absolutely exclude the sliding of nucleosomes during the preparation of chromatin, this result supports our interpretation of the precipitation experiments and may indicate that, in fact, topoisomerase I cleaves DNA bound to nucleosomes.

Nucleosome positioning is a critical determinant for the cleavages introduced by mammalian topoisomerase II into *in vitro* reconstituted SV40 chromatin (Capranico et al., 1990). In these studies, topoisomerase II cleavage sites were found to be strongly suppressed in DNA covered by nucleosomes and to persist or were even enhanced in linker DNA regions between individual nucleosomes.

In contrast, studies on the interaction of eukaryotic RNA polymerase II with nucleosomes have shown that the polymerase can bind to mononucleosomes and transcribe the DNA without apparent dissociation of histones. However, mononucleosomal particles as well as chromatin restrict binding of

the enzyme and the efficiency of DNA as a transcriptional template (Gonzales et al., 1989). These latter experiments are reminiscent of the results presented in this study and show that different enzymes can differ in their ability to recognize and to interact with DNA wrapped around histone octamer complexes.

Topoisomerase I interacts with protein-free DNA not at random but at specific sequences in the DNA. We have extended this observation with an analysis of topoisomerase I cleavage sites in mononucleosomes assembled according to two different methods. Our results indicate that the enzyme cleaves DNA in isolated nucleosomes with the same sequence specificity as protein-free DNA. It is known that camptothecin enhances topoisomerase I cleavages at certain sites in the DNA. Nucleosomes bound to DNA do not alter this property nor do they induce new cleavage sites or suppress sites present in protein-free DNA. Despite much effort to define the molecular determinants for topoisomerase I cleavage, a prediction of cleavage sites on the basis of established consensus sequences is not yet possible (Shen & Shen, 1990). Most likely, the local conformation within a particular DNA fragment and the nucleotide sequence will determine the cleavage site selection by topoisomerase I.

Topoisomerase I activity is affected by the superhelical density of the substrate DNA and the sequence of the DNA molecule. The *in vitro* studies presented in this paper further indicate that topoisomerase I mediated DNA cleavage is slightly suppressed when DNA is compared to chromatin as a substrate of topoisomerase I. Nucleosomes on DNA, however, neither exclude topoisomerase I generally from DNA nor do they influence the sequence recognition of the enzyme.

It is important to note that our nucleoprotein preparations do not contain some common chromatin proteins such as histone H1 or high mobility group (HMG) proteins. These proteins may affect the binding of topoisomerase I to chromatin and may influence the sequence recognition of the enzyme. These proteins may compete for DNA binding with topoisomerase I, thereby reducing the accessibility of DNA in certain chromosomal domains. However, they could even increase the interaction of the enzyme with DNA by direct protein-protein interactions as has been suggested for topoisomerase I stimulation by HMG 17 (Javaherian & Liu, 1983). Furthermore, the folding of chromatin in higher order structures certainly will influence the accessibility of these chromosomal regions for proteins and may exclude the DNA in these regions from recognition by topoisomerase I. In fact, actively transcribed chromosomal regions are enriched in topoisomerase I when compared to inactive chromatin (Weisbrod, 1982).

#### ACKNOWLEDGMENTS

We thank Dr. J. Kleinschmidt for providing the *X. laevis* oocyte extract and Drs. R. Knippers and W. Ruyechan for helpful discussions and critical reading of the manuscript. We thank Mrs. M. Kapitza for excellent technical assistance.

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

#### REFERENCES

- Ambrose, C., Lowman, H., Rajadhyaksha, A., Blasquez, V., & Bina, M. (1990) *J. Mol. Biol.* 214, 875-884.
- Andersen, A., Gocke, E., Bonven, J. B., Nielson, O., & Westergaard, O. (1985) *Nucleic Acids Res.* 13, 1543-1557.
- Austin, C. A., & Fisher, L. M. (1990) *Sci. Prog. (Oxford)* 74, 147-162.

- Been, M. D., Burgess, R. R., & Champoux, J. J. (1984) *Nucleic Acids Res.* 12, 3097-3114.
- Bonven, B. J., Gocke, E., & Westergaard, O. (1985) *Cell* 41, 541-551.
- Camilloni, G., DiMartino, E., DiMauro, E., & Caserta, M. (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86, 3080-3084.
- Capranico, G., Jaxel, C., Roberge, M., Kohn, K. W., & Pommier, Y. (1990) *Nucleic Acids Res.* 18, 4553-4559.
- Caserta, M., Amadei, A., DiMauro, E., & Camilloni, G. (1990) *Nucleic Acids Res.* 17, 8463-8474.
- Christiansen, K., Bonven, B. J., & Westergaard, O. (1987) *J. Mol. Biol.* 193, 517-525.
- Culotta, V., & Sollner-Webb, B. (1988) *Cell* 52, 585-597.
- Drew, H. R., & Travers, A. A. (1987) *J. Mol. Biol.* 186, 773-790.
- Edwards, K. A., Halligan, B. D., Davis, J. L., Nivera, N. L., & Liu, L. F. (1982) *Nucleic Acids Res.* 10, 2565-2576.
- Eng, W. K., Pandit, S. D., & Sternglanz, R. (1989) *J. Biol. Chem.* 264, 13373-13376.
- Giaever, G. N., Snyder, L., & Wang, J. C. (1988) *Biophys. Chem.* 29, 7-15.
- Gilmour, D. S., & Elgin, S. C. (1987) *Mol. Cell. Biol.* 7, 141-148.
- Gonzales, P. J., & Palacian, E. (1989) *J. Biol. Chem.* 264, 18457-18462.
- Hsieh, T. (1990) *Curr. Opin. Cell Biol.* 2, 461-463.
- Javaherian, K., & Liu, L. F. (1983) *Nucleic Acids Res.* 11, 461-472.
- Kjeldsen, E., Mollerup, S., Thomsen, B., Bonven, B. J., Bolund, L., & Westergaard, O. (1988) *J. Mol. Biol.* 202, 333-342.
- Kleinschmidt, J. A., & Franke, W. W. (1982) *Cell* 29, 799-809.
- Kunze, N., Klein, M., Richter, A., & Knippers, R. (1990) *Eur. J. Biochem.* 194, 323-330.
- Liu, L. F. (1989) *Annu. Rev. Biochem.* 58, 351-375.
- Lynn, R. M., Bjornsti, M. A., Caron, P. R., & Wang, J. C. (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86, 3559-3563.
- Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A laboratory manual*, Cold Spring Harbor Laboratory Cold Spring Harbor, NY.
- Maxwell, A., & Gellert, M. (1986) *Adv. Protein Chem.* 38, 69-107.
- Morse, R. H., & Simpson, R. T. (1988) *Cell* 54, 285-287.
- Ness, P. J., Koller, T., & Thoma, F. (1988) *J. Mol. Biol.* 200, 127-139.
- Perez-Stabel, C., Shen, C. C., & Shen, C. K. (1988) *Nucleic Acids Res.* 16, 7975-7993.
- Porter, S. E., & Champoux, J. J. (1989) *Nucleic Acids Res.* 17, 8521-8532.
- Richter, A., & Knippers, R. (1989) *Mol. Gen. (Life Sci. Adv.)* 8, 125-134.
- Richter, A., Strausfeld, U., & Knippers, R. (1987) *Nucleic Acids Res.* 15, 3455-3468.
- Rowe, T. C., Chen, G. L., Hsiang, Y. H., & Liu, L. F. (1986) *Cancer Res.* 46, 2021-2026.
- Rowe, T. C., Couto, E., & Kroll, D. J. (1987) *NCI Monographs* 4, 49-54.
- Shen, C. C., & Shen, C. K. (1990) *J. Mol. Biol.* 212, 67-78.
- Stewart, A. F., Herrera, R. E., & Nordheim, A. (1990) *Cell* 60, 141-149.
- Strausfeld, U., & Richter, A. (1989) *Prep. Biochem.* 19, 37-48.
- Sundin, O., & Varshavsky, A. (1980) *Cell* 21, 103-114.
- Trask, D. K., DiDonato, J. A., & Muller, M. T. (1984) *EMBO J.* 3, 671-676.
- Vosberg, H. P. (1985) *Curr. Top. Microbiol. Immunol.* 114, 19-102.
- Wang, J. C. (1985) *Annu. Rev. Biochem.* 54, 665-697.
- Wang, J. C. (1987) *Biochim. Biophys. Acta* 909, 1-9.
- Weisbrod, S. T. (1982) *Nucleic Acids Res.* 10, 2017-2042.